7

Biochemistry of fermentation

7.1 Introduction

Micro-organisms sustain their life cycles via a large number of interrelated/complex metabolic pathways covering both biosynthetic and energy-yielding functions. Each individual metabolic pathway consists of many reactions which, in turn, are regulated by different enzyme systems, and hence it is the level of enzyme synthesis and activity which maintains and controls the functions of the microbial cell (Stanier *et al*., 1987). One regulatory (or feedback) mechanism is derived from low molecular weight compounds which result from the breakdown of nutrients (carbohydrates, proteins, lipids and other minor constituents) present in the growth medium. The composition of this medium is, therefore, important in relation to the build-up and division of the microbial cells but, in the case of yoghurt, its effect on the metabolism and growth of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (including the "bio" cultures) also influences the properties and characteristics of the product. For this reason, the biochemical reactions initiated by the yoghurt and bio organisms are fundamental to the manufacture of a high quality product, and hence it is pertinent to consider them in some detail.

7.2 Carbohydrate metabolism

Microbial cells derive their energy requirements via different systems; the cytochrome system for harnessing energy from electrons of NADH, the enzymes that operate the anaplerotic pathways, the tricarboxylic acid cycle or by fermentation. The lactic acid bacteria (i.e. the lactococci, leuconostoc, lactobacilli, streptococci and bifidobacteria), however, do not possess any of the former three systems and energy can only be supplied by the fermentation of carbohydrates (Lawrence *et al*., 1976). The energy is largely obtained via substrate-level phosphorylation and the adenosine triphosphate enzymes (ATPases) of the cytoplasmic membrane (see also Nannen and Hutkins, 1991b). In general, dairy starter cultures metabolise car-

bohydrate (i.e. lactose is the main sugar present in milk) either through the homo- or heterofermentative metabolic pathways. *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* ferment lactose homofermentatively, whilst *Bifidobacterium* spp. ferment the same sugar heterofermentatively; the metabolic pathways of these micro-organisms are as follows.

7.2.1 Homolactic fermentation

Since the catabolism of lactose takes place inside the microbial cell, the key step in this metabolic pathway is at the entry of lactose into the cell. In the lactococci and certain strains of *L. acidophilus* (Kanatani and Oshimura, 1994; Marshall and Tamime, 1997a) a specific system is involved in lactose transport and the sugar is phosphorylated by phosphoenolpyruvate (PEP) during translocation by the PEP-dependent phosphotransferase system (PTS) as described by McKay *et al.* (1969) (see also Lawrence *et al.*, 1976). This mechanism is known as PEP:PTS and four proteins (in sequential order: enzyme II, III, I and HPr) are involved in translocating the lactose from outside to the inside of the cytoplasmic membrane and into the microbial cell to become lactose phosphate (Dills *et al.*, 1980; Zourari *et al.*, 1992b; Cogan and Hill, 1993; Monnet *et al.*, 1996). Lactose-6-phosphate is hydrolysed by β-phosphogalactosidase (β-Pgal) into its monosaccharide components. The galactose and glucose are then catabolised via the Tagatose and Emden–Meyerhof–Parnas EMP) pathways, respectively (Monnet *et al.*, 1996; Marshall and Tamime, 1997a). However, dephosphorylation of galactose may take place and it will remain unmetabolised and excreted from the microbial cell. Nevertheless, in both pathways the glucose and galactose converge at dihydroxyacetone phosphate and glyceraldehyde-3-phosphate where the three-carbon sugars become further oxidised to phosphoenolpyruvate and then produce lactic acid (see Fig. 7.1).

Homolactic fermentation by *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* follows the EMP pathway *mainly* for glucose catabolism. However, an alternative system for lactose transport into the cells of these starter cultures including *Bifidobacterium* spp. involves cytoplasmic proteins (permeases) that translocate lactose without chemical modification. Such a sugar transport mechanism could be similar to the lactose permease system in *Escherichia coli*. After the lactose enters the cell via a permease as an unphosphorylated disaccharide, it is hydrolysed by β-galactosidase (β-gal) to non-phosphorylated glucose and galactose. Glucose is catabolysed to pyruvate (see Fig. 7.1) and the galactose is secreted from the cell. When all the glucose is depleted, *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* will utilise the galactose via the Leloir pathway (Fig. 7.1) with galactokinase as the first enzyme of the metabolic pathway (Kandler, 1983; Hutkins *et al.*, 1985b; Hickey *et al.*, 1986; Thompson, 1988; Benito de Cardenas *et al.*, 1989; Poolman *et al.*, 1989, 1990, 1995; le Bras *et al.*, 1991; Hutkins and Ponne, 1991; Collins and Thompson, 1992; Zourari *et al.*, 1992a; Poolman, 1993). However, Cogan and Hill (1993) suggest that some strains can metabolise galactose only when low (4 mM) concentrations of lactose are present; this may be due to an antiporter proton motive force (PMF) transport system involving galactose, but the details have yet to be established (see also Thomas and Crow, 1984; Hutkins *et al.*, 1985a).

Thus, it appears the carbohydrate metabolism by *S. thermophilus* differs from lactococcal species. Characterisation of the metabolic activity of this organism has been

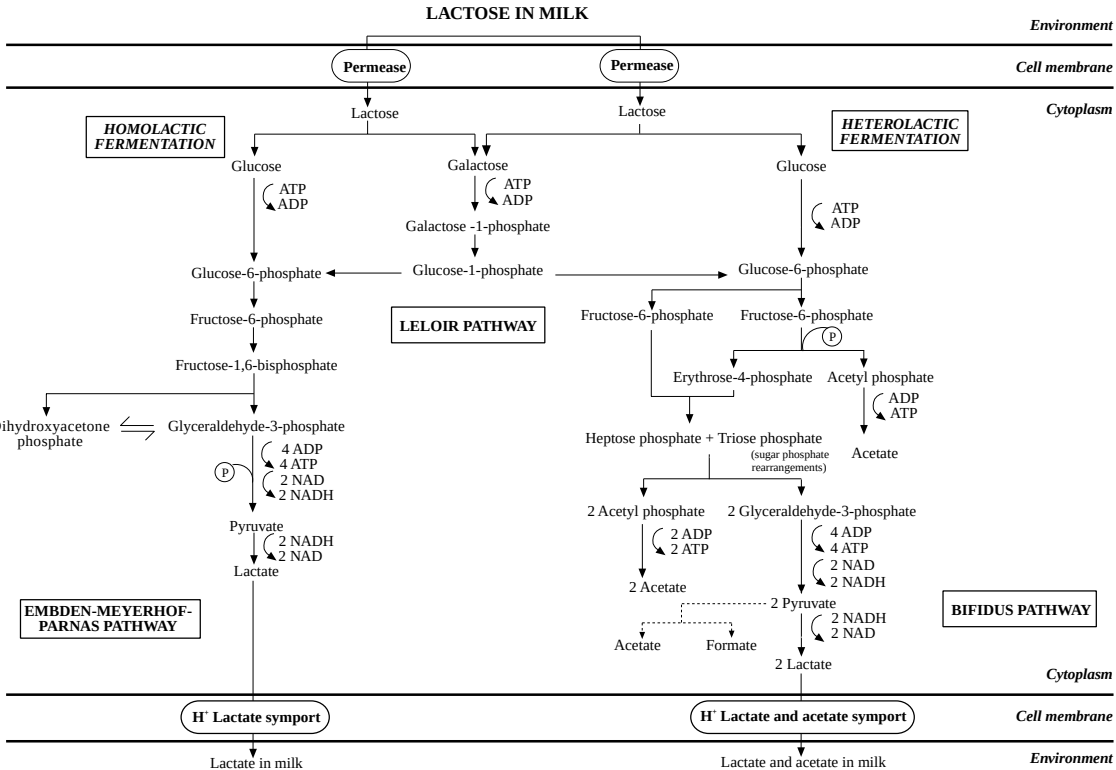


Fig. 7.1 Homolactic and heterolactic fermentation of lactose by the yoghurt and bio starter cultures after translocation by a permease

Note: The dotted line sequence may indicate an alternative pathway to account for excess acetate observed in fermentation by some *Bifidobacterium* strains.

Adapted from Monnet *et al*. (1996) and Marshall and Tamime (1997a).

reported by Hemme and Nardi (1980), Hemme *et al.* (1980) and in the reviews by Hutkins and Morris (1987), Ramos and Harlander (1990) and Arihara and Luchansky (1995).

It is worth pointing out that the presence of CO_2 during the fermentation of milk stimulates the growth of *L. delbrueckii* subsp. *bulgaricus* (see Chapter 6) and, if it is accepted that *S. thermophilus* can metabolise galactose via the Leloir pathway, this may explain the presence of CO_2 in the milk (see Fig. 7.1); however, an alternative route for the production of CO_2 is the hydrolysis of urea (Tinson *et al.*, 1982a–c).

Lactate dehydrogenase is also important in the control of carbohydrate metabolism. The enzyme in *Lactococcus* spp. is activated by fructose 1,6-bisphosphate aldolase and by tagtose 1,2-bisphosphate aldolase (see the reviews by Monnet *et al.*, 1996; Marshall and Tamime, 1997a). The homolactic fermentation of *Lactobacillus* spp. may be different, as the enzyme from many species has been found to have constitutively high activity which is independent of the presence of fructose 1,6-bisphosphate aldolase. Sequencing the lactate dehydrogenase gene from *S. thermophilus* shows it to have 328 amino acid residues (Taguchi and Ohta, 1991, 1993), whilst 332 amino acid residues were reported by Kochhar *et al.* (1992d) for the equivalent gene from *L. delbrueckii* subsp. *bulgaricus* (see also Kochhar *et al.*, 1992a–c). Branny *et al.* (1996) observed that the gene encoding for pyruvate kinase and for phosphofructokinase from *L. delbrueckii* subsp. *bulgaricus* formed a bicistronic operon transcribed into 2.9 kb RNA (see also Branny *et al.*, 1998). Somkuti and Steinberg (1991) reported that sucrose (suc^-) mutants strains isolated after treating *S. thermophilus* with *N*-methyl-*N*-nitroso-*N*′-nitroguanidine were able to utilise lactose, but not sucrose, and retained the ability to synthesise β-fructofuranosidase (see also Hosono *et al.*, 1989); characterisation of a Mn-containing superoxide dismutase in *S. thermophilus* has been reported by Chang and Hassan (1997). Details of further aspects of sugar metabolism and the synthesis of L(+) and D(−) lactic acid by the yoghurt micro-organisms isolated from commercial products in Argentina, South Africa and Canada have been given by Malan (1987), Amoroso *et al.* (1988, 1989, 1992), Sinha *et al.* (1989) and Amoroso and Manca de Nadra (1991) (see also Richmond *et al.*, 1987).

7.2.2 Heterolactic fermentation

In the present context, only the bifidobacteria ferment lactose and glucose via a heterofermentative pathway (Fig. 7.1). The catabolism of glucose produces no CO_2 because there is no early step involving a decarboxylation. As mentioned earlier, lactose is transported into the cell by means of permease and, in turn, it is hydrolysed into glucose and galactose. Aldolase and glucose-6-phosphate dehydrogenase are absent in this species. Hexoses are catabolised by a fructose-6-phosphate shunt and the pathway involves fructose-6-phosphate phosphoketolase. The products of fermentation by *Bifidobacterium* spp. are lactate and acetate, and the fermentation of two molecules of glucose yields three molecules of acetate and two molecules of lactate.

7.2.3 Lactase activity

β-Galactosidase from the yoghurt organisms has been identified as an important enzyme in fermented milk processing and is mainly involved in lactose catabolism.

However, the enzyme from *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* has also been characterised as an alternative source of lactase for commercial developments.

The optimum activity of streptococcal β-gal has been observed as follows: (a) neutral pH, (b) temperature at 55°C in buffer, (c) more heat stable than a similar enzyme from yeasts, (d) Mg^{2+} stimulated enzyme activity, whilst EDTA caused inhibition, and (e) the presence of oxgall (0.15 ml 100 ml^{-1}) increased the activity of β-gal (Greenberg and Mahoney, 1982; Noh and Gilliland, 1994; Garman *et al*., 1996; Gündüz and Rejaee, 1997). Greenberg and Mahoney (1984) observed that the activity of the enzyme was greater in heated (63°C or 85°C for 30 min) milk than in raw milk, whilst the activity in a buffered system was greater than in whey or milk, due to the unfavourable ionic environment in the latter. The stability of β-gal in milk and sweet whey was ≥10-fold that in lactose solution (Greenberg *et al*., 1985). However, thermal denaturation occurs at ~60°C, but stability can be enhanced by the addition of bovine serum albumin (Chang and Mahoney, 1994). In milk the activation energy for lactose hydrolysis was 35 kJ mol^{-1} (Chang and Mahoney, 1989a, b); different strains of *S. thermophilus* demonstrate different β-gal activities (Occhino *et al*., 1986).

One possible use of β-gal from *S. thermophilus* is the hydrolysis of lactose in milk without concomitant production of lactic acid (Somkuti and Steinberg, 1995; see also Smart *et al*., 1985; Smart and Richardson, 1987; Smart, 1991; Benateya *et al*., 1991; Linko *et al*., 1998) or the immobilisation of β-gal on DEAE-cellulose for the production of low lactose milk (Sharma and Dutta, 1990). From genetic studies (David *et al*., 1992), the β-gal of the yoghurt organisms and *Leuconostoc lactis* are similar, and *in vivo* activity of β-gal in high lactose yoghurt was much less acid resistant than that in ordinary yoghurt (Kotz *et al*., 1994); the β-gal activities of three commercial bio yoghurts were reported by Ordonez and Jeon (1995).

The enzyme from *L. delbrueckii* subsp. *bulgaricus* may have a requirement for Mg^{2+} for activity (Adams *et al*., 1994) and a pH optimum of 6.5–7.0, although the enzyme is stable at pH 5.8 (Gupta *et al*., 1994). The β-galactosidase of *L. delbrueckii* subsp. *bulgaricus* is made of a dimer consisting of two subunits of identical size (molecular weight 235 kDa). It was rapidly and irreversibly inactivated at pH 4 due to a decline in the number of the exposed tryptophan residues because of the denaturation process (Winters and Batt, 1991). The optimum activity (i.e. pH 7 and 55°C) of the enzyme in autoclaved milk resulted in ~85% of the lactose being hydrolysed (Shah and Jelen, 1991; see also Yoast *et al*., 1994). The specific activity of β-gal was greater in lactobacilli in group I (i.e. *Thermobacterium*) than in group II (i.e. *Streptobacterium*) (Cesca *et al*., 1984) and Nader de Macias *et al*. (1986) used the enzyme-linked immunosorbent assay (ELISA) for determining the immunological relationships among β-gal from different lactobacilli (see also Wang *et al*., 1996) or for arginine dihydrolase activity in the lactobacilli groups I, II and III (Manca de Nadra *et al*., 1982).

β-Phosphogalactosidase (β-Pgal) has been reported in *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Permi *et al*., 1972; Reddy *et al*., 1973b; Somkuti and Steinberg, 1978, 1979a, b; Farrow, 1980; Toba *et al*., 1981) so that galactose could be metabolised. However, Cogan and Hill (1993) reported that the enzyme is an artefact derived by formation of *o*-nitrophenyl-β-galactopyranoside (ONPG), the substrate for β-gal, from ONPG phosphate, the substrate for β-Pgal, by a phosphatase.

7.2.4 Production of lactic acid

The catabolism of lactose by *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and bifidobacteria results mainly in the production of lactic acid, or lactic and acetic acids when bifidobacteria are used in the starter culture (see Fig. 7.1). Although the conversion process consists of many different biochemical reactions it can be simplified by the following equation:

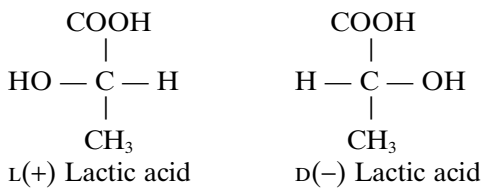
$$\begin{array}{lcll} \text{Lactose} & + \text{Water} & \rightarrow & \text{Lactic acid} \\ C_{12}H_{22}O_{11} & H_2O & & 4C_3H_6O_3 \end{array}$$

Lactic acid is important during the manufacture of yoghurt for the following reasons.

First, the lactic acid helps to destabilise the casein micelles by progressively converting the colloidal calcium/phosphate complex (in the micelle) to the soluble calcium phosphate fraction which diffuses into the aqueous phase of the milk. This results in the micelles being gradually depleted of calcium, so leading to coagulation of the casein at pH 4.6–4.7 and the formation of the yoghurt gel (refer to Chapter 2 for further details). Once this physical condition has been established, soluble calcium lactate is formed and according to Dyachenko (1971) the destabilisation reaction can be summarised as follows:

$$\begin{array}{lcl} \text{Ca-caseinate-phosphate complex} + \text{lactic acid} & \rightarrow & \text{Casein complex} \\ & & + \\ & & \text{Ca-lactate} \\ & & + \\ & & \text{Ca-phosphate} \end{array}$$

Second, the lactic acid gives yoghurt its distinctive and characteristic taste (i.e. sharp and acidic). It can also enhance or contribute to the nutty and/or aromatic flavour of the product. Lactic acid bacteria possess the enzyme lactate dehydrogenase (LDH) for the synthesis of lactate from pyruvate (see Fig. 7.1). Lactate is the Latin word for lactic acid derived from milk. Different forms of lactic acid can be produced (e.g. L(+), D(−) or DL(±)) and these isomers differ in the configuration of the second carbon atom, as follows:



In yoghurt starter cultures, *S. thermophilus* produces mainly L(+) lactic acid (Garvie, 1978; Hemme *et al.*, 1981) and D(−) lactic acid is produced by *L. delbrueckii* subsp. *bulgaricus* (Gasser, 1970; Gasser and Gasser, 1971; see also the review by Tamime and Deeth, 1980). The LDH enzyme is situated in the cytoplasm of the bacterial cell and according to Garvie (1980) the activity of this enzyme is, in the yoghurt organisms, dependent on nicotinamide adenine dinucleotide (NAD)/reduced nicotinamide adenine dinucleotide (NADH). The former coenzyme is regenerated from NADH during the conversion of pyruvic acid to lactic acid. However, some strains of *S. thermophilus* contain an LDH enzyme which is activated by fructose 1,6-

bisphosphate (FDP) (Wolin, 1964; Garvie, 1980) and such enzymes show an absolute requirement for FDP at physiological pHs; the reaction is virtually non-reversible and the enzyme reacts weakly with lactic acid and NAD (see also Delcour *et al*., 1993; Bernard *et al*., 1994, 1995, 1997; Álvarez *et al*., 1997).

Recently, Vinals *et al*. (1995) described the structure of LDH of *L. delbrueckii* subsp. *bulgaricus* as being constituted of subunits of α/β structure with a catalytic domain (i.e. consisting of a histidine residue along with arginine and phenyalanine) and a coenzyme binding domain.

During the manufacture of yoghurt, *S. thermophilus* grows faster than *L. delbrueckii* subsp. *bulgaricus* (see Fig. 6.2), and hence L(+) lactic acid is produced first followed by D(−) lactic acid. The percentage of each isomer present in yoghurt is an indication of the following:

- Yoghurt, which contains more than 70% of L(+) lactic acid has been inoculated with a starter culture which consists predominantly of *S. thermophilus* (Kunath and von Kandler, 1980), or the fermentation has been carried out at a temperature below 40°C, or the product has been cooled to a low acidity and the cooled yoghurt contains around $0.8\,g\,100\,ml^{-1}$ or less lactic acid.
- Yoghurt containing more D(−) lactic acid than L(+) lactic acid has been incubated at too high a temperature, i.e. 45°C or more, or for a long period whereby the product has become highly acidic, or has suffered from prolonged storage, or the starter inoculation rate was more than 3%, or the starter contained more rods than cocci.

Yoghurt usually contains 45–60% L(+) lactic acid and 40–55% D(−) lactic acid (Puhan *et al*., 1973a, b, 1974; Vanderpoorten and von Renterghem, 1974; Kielwein and Daun, 1980; Aleksieva *et al*., 1981), and the ratio of L(+):D(−) lactic acid could be used to assess the quality of yoghurt. However, Puhan *et al*. (1973b, 1974) examined 269 samples of commercial yoghurt and found that the ratio of L(+):D(−) ranged from as little as 0.34 (very acidic) to 8.28 (i.e. L(+) lactic acid predominant). A ratio of two was suggested by Blumenthal and Helbling (1974) to be consistent with a good yoghurt, but such an approach could be more useful in situations where the quality of yoghurt (i.e. sweet-low in acid or sharp-high in acid) has to be manipulated to meet the demands of consumers in different markets, that is, a sharp and acidic yoghurt must contain a low ratio of L(+):D(−) and vice versa. A combined starter of *Lactobacillus helveticus* and *S. thermophilus* used for the manufacture of yana yoghurt in Bulgaria gave rise to >80% L(+) lactic acid which is suitable for infant foods (Gyosheva *et al*., 1996). A similar result was obtained by reducing the lactate dehydrogenase activity in *L. delbrueckii* subsp. *bulgaricus* (Germond *et al*., 1995) (see also Klupsch, 1984).

L. acidophilus produces DL lactic acid, whilst the bifidobacteria produces L(+) acid as the result of lactose metabolism. Marshall and Tamime (1997b) have shown that these organisms do not produce acid at the same rate as *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Furthermore, most of the bio starter cultures rely on the yoghurt organisms (singly or mixed) for the acidification of milk and hence it was decided not to review the metabolism of bio organisms in detail. One aspect which should not be overlooked, however, is the amount of acetic acid produced in bio yoghurt. High levels will impart a "vinegary" taste which may not be accepted by consumers (see Fig. 7.1).

Nevertheless, the main role of the yoghurt organisms is to acidify the milk by producing lactic acid from lactose. Detailed information on the anaerobic fermentation reacts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (i.e. as single or mixed strains including different strains of each species) has been reported by Oner and Erickson (1986), Oner *et al*. (1986a–d), Zourari and Desmazeaud (1990, 1991) and Zourari *et al*. (1991). Such characterisation of yoghurt organisms is aimed at blending strains together so that different products can be made for different markets and can include variations in flavour and aroma (see also Nannen and Hutkins, 1991a; Zanatta and Basso, 1992; Hutkins and Nannen, 1993). In addition, it has been reported that the undenatured whey protein in skimmed milk decreased during the incubation period with the yoghurt starter cultures (Vaitheeswaran and Bhat, 1988).

7.2.5 Production of exopolysaccharide (EPS)

Some strains of bacteria utilise the carbohydrates in the growth medium for the production of EPS materials, and examples of such organisms are *Streptococcus mutans*, *Streptococcus bovis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* which have the ability to produce extracellular dextrans (Berkeley *et al.*, 1979). Sharpe *et al*. (1972) isolated a similar material, slime, from some heterofermentative *Lactobacillus* spp. and it was found to be a glucan, probably dextran, consisting of α-1-6-glycosidic linkages. At present, isolated strains of *Lactococcus* spp. and thermophilic lactic acid bacteria are used extensively in the manufacture of fermented milks and many EPS-producing lactic acid bacteria have been studied extensively since 1990 (see the reviews by Cerning, 1990, 1994, 1995; Malik *et al*., 1994; Sikkema and Oba, 1998). The role of EPS in the consistency and texture of yoghurt has been discussed elsewhere (Chapter 2 and 10; see also Wacher-Rodarte *et al*., 1993; Uemura *et al*., 1994; Giraffa, 1994; Lira *et al*., 1997; Rawson and Marshall, 1997; Sebastiani and Zelger, 1998).

EPS materials are produced by some yoghurt starter cultures, for example, the RR culture which was developed in The Netherlands to enhance the viscosity of yoghurt (Galesloot and Hassing, 1966; see also Tamime and Robinson, 1978; Luczynska *et al.*, 1978). The work of Tamime (1977a, b, 1978) suggested that the chemical composition of the EPS material produced by starter culture RR was a β-glucan which yielded only glucose after acid hydrolysis. However, current studies suggest that the yield and carbohydrate constituents of the EPS materials produced by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are influenced by many factors such as the growth medium used, the temperature of incubation, the level of acidity in the growth medium and the strain variation (Cerning 1990, 1994, 1995; Petit *et al*., 1991; Gassem *et al*., 1995, 1997a, b; Grobben *et al*., 1995, 1997, 1998; Mollet, 1996).

In general, the amount of EPS material produced by the yoghurt organisms may reach up to 40 mg 100 ml^{-1} (Cerning, 1995). Further factors relating to yield and production of EPS are summarised in Table 7.1. No data are available on EPS production by the bio starter cultures. However, Mozzi *et al*. (1995a) reported that optimum yield of EPS from a strain of *L. acidophilus* was ~6 mg 100 ml^{-1} after incubation at 37°C or 42°C for 24 hours.

It is evident that a number of strains of the yoghurt starter culture are capable of producing EPS. These are classified as heteropolysaccharides composed of either

Table 7.1 Reported factors that can influence the yield and characteristic of EPS by the yoghurt organism

Micro-organism	Comments	References
S. thermophilus	Ropy strains exhibited an increase in viscosity in milk, but not in whey or synthetic media; they produced more soluble polysaccharides ($\leq 4\,mg\,100\,ml^{-1}$) than the non-ropy strains; when grown at 30°C, the ropy strains produced four to eight times more insoluble glucides than the non-ropy strains.	Giraffa and Bergère (1987)
	The amount of EPS produced ranged between 5 and $34\,mg\,100\,ml^{-1}$ in UF milk enriched with casamino acid or heart extract; compared with skimmed milk, the amount of EPS produced was much lower.	Cerning *et al*. (1990)
	Grown in synthetic media, EPS was produced in the stationary phase; factors that influenced EPS production were type of sugar, temperature and initial pH; at optimal growth rate, EPS production was dependent on lactose concentration.	Gancel and Novel (1994a, b)
	Optimum yield of EPS ($\sim 10\,mg\,100\,ml^{-1}$) was obtained when the organism was incubated at 30°C for 24 hour.	Mozzi *et al*. (1995c)
L. delbrueckii subsp. *bulgaricus*	Isolates from a commercial yoghurt produced soluble EPS.	Manca de Nadra *et al*. (1985)
	Yield of EPS ranged between 6 and $43\,mg\,100\,ml^{-1}$; growth media did not influence amount of EPS produced.	Cerning *et al*. (1990)
	At higher temperature and slower growth, the EPS production per cell was greater; EPS production was increased in the presence of hydrolysed casein early in the growth phase when grown in milk, but was reduced in MRS broth and lactose; preliminary results suggested that the EPS is a glycoprotein, although the protein may be loosely associated with the carbohydrates.	Garcia-Garibay and Marshall (1991)
	Yield of $12\,mg\,100\,ml^{-1}$ was optimal when the organism was grown at 37°C for 24 hours.	Mozzi *et al*. (1995c)
	Half the amount of EPS was produced in the exponential phase; the yield of EPS in skimmed milk reached $13\,mg\,100\,ml^{-1}$.	Bouzar *et al.* (1996)
	Glucose + fructose influenced the yield of EPS and produced $8\,mg\,100\,ml^{-1}$ which was the highest.	Grobben *et al*. (1996, 1997)
	EPS yield was $35.4\,mg\,100\,ml^{-1}$.	Kimmel *et al*. (1998)
Mixed culture	Excessive EPS production when the starter culture was incubated at 32°C for a long time; such yoghurt had a coagulum with decreased relative firmness and apparent viscosity.	Schellaas (1984), Schellaas and Morris (1985)
	A yield of $80\,mg\,100\,ml^{-1}$ was obtained when both cultures used were EPS producers.	Cerning *et al*. (1990)
	Growth of lactobacilli (EPS producer) with streptococci (non EPS producer) yielded $24\,mg\,ml^{-1}$ of EPS in skimmed milk.	Bouzar *et al*. (1997)

linear or branched repeating units varying in size from di- to heptasaccharides. The molecular weights of the EPS are rather high ranging from $1–2 \times 10^6$ which is formed by polymerisation of hundreds and possibly thousands of these repeating units. The available data suggest a range of different EPS structures:

- In some mixed cultures, the EPS structure consists of galactose and glucose at a ratio of 2:1 (Schellhaas, 1984; Schellhaas and Morris, 1985), but a ratio of 1:1 was reported by Lemoine *et al.* (1997) for *S. thermophilus*. However, the EPS material produced by *L. delbrueckii* subsp. *bulgaricus* consisted of glucose and fructose (ratio 1:2) and the predominant linkages were α-1,4 and α-1,6-glucosidic linkages at a ratio of 1:1 (Manca de Nadra *et al.*, 1985).
- EPS produced by *L. delbrueckii* subsp. *bulgaricus* consisted of galactose, glucose and rhamnose at a ratio of 4:1:1 (Cerning *et al.*, 1986), 5:1:3 (Gruter *et al.*, 1993) or 7:1:0.8 (Grobben *et al.*, 1996); however, Lemoine *et al.* (1997) reported a ratio of 3:1:2 for EPS produced by *S. thermophilus*.
- *L. delbrueckii* subsp. *bulgaricus* produced EPS made up of glucose and galactose with small amounts of mannose (Bouzar *et al.*, 1996) or mainly galactose and small amounts of glucose and rhamnose (Bouzar *et al.*, 1997; see also Zourari *et al.*, 1992a).
- Cerning *et al.* (1988) reported that glucose and galactose were the main saccharides of the EPS material from *S. thermophilus*, along with small amounts of xylose, arabinose, rhamnose and mannose; whilst Ariga *et al.* (1992) reported a ratio of 1:1.47 of rhamnose and galactose in an EPS produced by the same organism.

Nevertheless, the structures of the EPS produced by some yoghurt organisms have been determined by Doco *et al.* (1990, 1991), Gruter *et al.* (1993), Stingele *et al.* (1996) and Lemoine *et al.* (1997). The polymers are based on D-galactose residues connected via 1 → 3 or 1 → 4 glycosidic linkages as follows:
S. thermophilus (i.e. tetrasaccharide)

[→3)-β-D-Gal*p*-(1→3)-[α-D-Gal*p*-(1→6)]-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1→]*n*
[→3)[α-D-Glc*p*-(1→3)-[β-D-Gal*p*-(1→6)]-β-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→]*n*
[→2)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→4)]-α-L-Rha*p*-(1→]*n*

L. delbrueckii subsp. *bulgaricus* (i.e. branched heptasaccharide)

[→2)-[β-D-Gal*p*-(1→3)]-α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)]-[α-L-Rha*p*-(1→3)]-α-D-Gal*p*-(1→]*n*

where Gal*p* is galactopyranose, Gal*f* is galactofuranose, Glc*p* is glucopyranose, Rha*p* is rhamnopyranose and NAc is *N*-acetyl-D-galactosamine.

In some strains, EPS production is sometimes unstable (e.g. in *S. thermophilus*) due, perhaps, to the presence of glycohydrolase capable of hydrolysing the EPS material (Zourari *et al.*, 1992a).

Other structures of EPS produced by lactic acid bacteria have been reported and some typical examples are *Lactococcus lactis* subsp. *cremoris* (Nakajima *et al.*, 1990; Cerning *et al.*, 1992; Gruter *et al.*, 1992), *Lactobacillus paracasei* subsp. *paracasei* (Robijn *et al.*, 1996; Mozzi *et al.*, 1994, 1995b, c, 1996, 1997), *Lactobacillus helveticus*

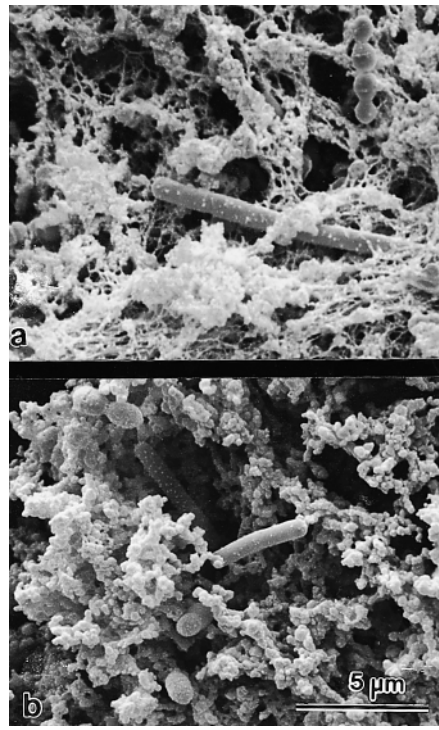


Fig. 7.2 The microstructure (SEM) of stirred yoghurt made with (a) and without (b) EPS starter cultures

After Skriver *et al.* (1995). Reproduced with permission of *Milchwissenschaft*.

(Robijn *et al.*, 1995a), *Lactobacillus sake* (Robijn *et al.*, 1995b), *Bifidobacterium longum* (Roberts *et al.*, 1995; Andaloussi *et al.*, 1995) and *Lactobacillus rhamnosus* (Gamar *et al.*, 1997). However, little is known about the metabolic synthesis of EPS material produced by lactic acid bacteria, including the factors that trigger the mechanisms in the microbial cell. Some hypotheses or possible routes for the synthesis of EPS have been reported by Suzuki (1990), Grobben *et al.* (1996), Stingele *et al.* (1996) and Escalante *et al.* (1998), whilst other researchers have patented EPS producing starter cultures for the manufacture of fermented milks (Vedamuthu, 1982; Gancel *et al.*, 1989; Doco *et al.*, 1989).

As already discussed in Chapter 2, the microstructure of yoghurt consists of a protein matrix composed of casein micelle chains and clusters and the fat globules are embedded in the protein matrix. The production of EPS by the yoghurt starter organisms results in a web of filaments attaching the microbial cell to the protein matrix of the yoghurt (Tamime *et al.*, 1984; Schellaas and Morris, 1985; Bottazzi and Bianchi, 1986; Skriver *et al.*, 1995). However, Skriver *et al.* (1995) reported that the attachment of the filaments to the bacterial cells and the protein could be influenced by the type of yoghurt produced. Figure 7.2 shows such an effect in set-type yoghurt (see also Teggatz and Morris, 1990). The microstructure of stirred yoghurt made at two different laboratories is somewhat different, in that the attachment of these filaments between the microbial cells was not evident, but

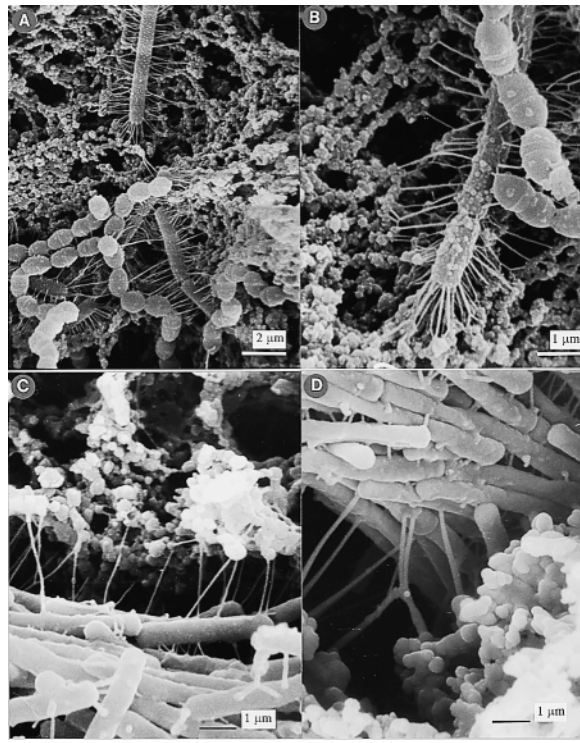


Fig. 7.3 EPS production by *L. delbrueckii* subsp. *bulgaricus* in yoghurt made at different laboratories

A and B, Tamime and Kalab (Scotland and Canada); C and D, Bottazzi (Italy).

they formed links between the casein micelles of the protein matrix (see Fig. 7.3). Such minor changes in the microstructure of the yoghurt could be attributed to mechanical effects that disrupted the attachment of the EPS to the microbial cell (Skriver *et al*., 1995).

It is evident that some technical data are available on EPS production by the lactic acid bacteria, but more information is still awaited on the mechanisms that control the anabolic characteristics. For example, what triggers the starter culture to polymerise sugars instead of breaking them down for use as an energy source?

7.2.6 Production of flavour compounds

Starter cultures are primarily responsible for the production of the flavour compounds which contribute to the aroma of yoghurt. These compounds may be divided into four main categories:

- Non-volatile acids (lactic, pyruvic, oxalic or succinic)
- Volatile acids (formic, acetic, propionic or butyric)
- Carbonyl compounds (acetaldehyde, acetone, acetoin or diacetyl)
- Miscellaneous compounds (certain amino acids and/or constituents formed by thermal degradation of protein, fat or lactose).

Table 7.2 Production of carbonyl compounds ($\mu g\,g^{-1}$) by yoghurt starter cultures

Organism	Acetaldehyde	Acetone	Acetoin	Diacetyl
S. thermophilus	1.0–13.5	0.2–5.2	1.5–7.0	0.1–13.0
L. delbrueckii subsp. *bulgaricus*	1.4–77.5	0.3–3.2	Trace–2.0	0.5–13.0
Mixed cultures	2.0–41.0	1.3–4.0	2.2–5.7	0.4–0.9

Data compiled from Tamime and Deeth (1980), Abo-Elnaga and Hegazi (1981), Singh *et al.* (1982), Yaygin (1982b), Abou-Donia *et al.* (1984), Ashour *et al.* (1985), Yu and Chung (1986), Hegazi and Abo-Elnaga (1989, 1990), Kneifel *et al.* (1992) and Thomopoulos *et al.* (1993).

There is general agreement in the literature that the aroma and flavour of yoghurt are basically due to the production of non-volatile and volatile acids and carbonyl compounds. For further detail refer to the reviews by Adda (1986), Marshall (1987), Mogensen (1992), Fernandez-Garcia and McGregor (1994), Cogan (1995) and Marshall and Tamime (1997a). Pette and Lolkema (1950) were the first to investigate the flavour of yoghurt and they concluded that the aroma was due to the presence of acetaldehyde and other unidentifiable compounds; however, they also observed that the level of acetaldehyde was much greater in mixed cultures due to the associative growth of the yoghurt organisms, although *L. delbrueckii* subsp. *bulgaricus* played the more important role. This observation has been confirmed by many workers and a summary of these results can be seen in Table 7.2.

Organoleptic assessments of yoghurt by Pette and Lolkema (1950) and Schulz and Hingst (1954) showed that yoghurt was rated best or high by a taste panel when the product contained a low level of acetaldehyde, and they suggested that other carbonyl compounds may be primarily responsible for the typical yoghurt flavour and/or aroma. This view was shared by Bottazzi and Dellaglio (1967) who observed that single strains of *S. thermophilus* produced equal quantities of acetaldehyde and diacetyl, and that a ratio of 1:1 of these compounds typifies the desired aroma of yoghurt. However, in another publication from the same laboratory, Bottazzi and Vescovo (1969) attributed a fullness of yoghurt flavour to a ratio of 2.8:1 of acetaldehyde to acetone, both of which were produced by single cultures of *S. thermophilus*; only a small amount of acetone was produced by *L. delbrueckii* subsp. *bulgaricus.* Incidentally, the same workers did not observe any diacetyl production by these particular test organisms, whereas Dutta *et al.* (1973) obtained $13\,\mu g\,g^{-1}$ of diacetyl (the highest level reported in the literature) from single strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (see also Baisya and Bose, 1975; Mutai *et al.*, 1972). The production of such high levels of diacetyl and acetoin by single cultures does not appear to correspond with the reported levels of these compounds in yoghurt (see Table 7.2). These discrepancies could be attributed to variations in the strains of streptococci and lactobacilli used, or to differences in the analytical methods employed to detect the level of these carbonyl compounds, and/or to alterations in the level of milk solids, type of milk and degree of heat treatment used during the preparation of the milk base (see Robinson *et al.*, 1977; Tamime, 1977a, b; Yaygin, 1982a; Schmidt *et al.*, 1983; Ulberth, 1991; Kneifel *et al.*, 1992).

Table 7.3 Detectable levels of acetaldehyde in yoghurt produced with different starter cultures

Starter culture	Acetaldehyde	
	($\mu g\,g^{-1}$)	Mean differences
CH-1	37.5	±2.3
Boll-3	27.6	±1.3
RR	10.4	±0.3

A colorimetric test method was used which was non-specific for acetaldehyde as it measured the total content of ketones and aldehyde constituents.

Figures are the mean of 10 samples and the acidity ranged from pH 4.0–4.1 or 1.1–1.2 g $100\,g^{-1}$ lactic acid.

After Tamime (1977a) and Robinson *et al.* (1977).

It could be argued, of course, that the presence of these carbonyl compounds is not essential, for instance, in fruit and flavoured yoghurts, but a high level of acetaldehyde is desirable for the typical aroma of natural or plain yoghurt and Suzuki *et al.* (1979) concluded that yoghurt, which contained only $7\,\mu g\,g^{-1}$ acetaldehyde, did not have sufficient of the desirable yoghurt flavour. Furthermore, the same workers detected high levels of diacetyl in fermented milks only in the presence of *Lactococcus lactis* biovar *diacetylactis*, a view supported by many authors (see Table 7.2 for level of diacetyl production by mixed yoghurt cultures and Chapter 5 for the production of dahi).

Robinson *et al.* (1977) and Tamime (1977a) assessed, both organoleptically and for the presence of carbonyl compounds, samples of natural yoghurt made using different strains of starter culture (CH-1 (normal), Boll-3 (viscous) and RR (EPS producer) – the former two cultures were obtained from Chr. Hansen's Lab. A/S, Denmark and culture (RR) from NIZO, The Netherlands). The judging panel consisted of Mediterranean and non-Mediterranean nationalities. The preference trend was for yoghurt made by culture (CH-1) (i.e. sharp and acidic), followed by (Boll-3), and the least preferred, especially by the Mediterranean nationalities, was the yoghurt made by starter (RR). The level of acetaldehyde in these yoghurts is illustrated in Table 7.3, where it can be observed that starter culture (CH-1) produced the highest level of acetaldehyde, followed by (Boll-3) and finally (RR). Hence, these results tend to confirm that the typical aroma and flavour of natural or plain yoghurt is directly associated with the presence of carbonyl compounds, mainly acetaldehyde, in the product.

Aroma profiles and sensory studies have been correlated with a view to classifying yoghurt made with different starter cultures in Bulgaria by Gyosheva (1985) and Beshkova *et al.* (1998) and in Austria by Ulberth and Kneifel (1992). The aroma compounds that were identified in typical Bulgarian yoghurts were acetaldehyde, acetone, ethyl acetate, butanone, diacetyl and ethanol (Kondratenko and Gyosheva, 1985; Gyosheva, 1986). However, in Switzerland, 91 components were identified in yoghurt, of which 21 had a major impact on the flavour (Ott *et al.*, 1997; see also Reps *et al.*, 1987; Laye *et al.*, 1993; Imhof *et al.*, 1994, 1995).

During the manufacture of yoghurt, the production of acetaldehyde becomes evident only at a certain level of acidification (i.e. pH 5.0), reaches a maximum at pH 4.2 and stabilises at pH 4.0. Fortification of the milk base with milk solids, and certain heat treatments of the yoghurt milk, can significantly increase the acetaldehyde content of the yoghurt (Gorner *et al.*, 1968). In acidified milk products, the partition coefficients (i.e. between air and aqueous phases) of carbonyl compounds (acetaldehyde and diacetyl) and ethanol were higher at 50°C than at 30°C, and increased as the solids-not-fat (SNF) ($12\,g\,100\,g^{-1}$) and fat ($20\,g\,100\,g^{-1}$) concentrations increased in the milk base (Lee *et al.*, 1995); the pattern of partition coefficients was acetaldehyde > diacetyl > ethanol. The production of diacetyl and acetoin in fresh milk (cow's or buffalo's) was more than in reconstituted dried whole milk (Ismail *et al.*, 1980). However, comparative studies of flavour development are limited; for example, more volatile acids were found in goat's milk than in cow's milk, whilst more acetaldehyde was produced by the yoghurt starter cultures in cow's milk than in goat's milk (Manjunath *et al.*, 1983; Rysstad and Abrahamsen, 1987). Yaygin (1982a) and Yaygin and Mehanna (1988) reported the contents ($\mu g\,g^{-1}$) of aroma compounds (i.e. range) in yoghurt made from different mammalian milks as follows:

Milk	Acetaldehyde	Acetone	Ethanol
Cow	4–26	3–25	19–365
Sheep	7–30	5–30	10–255
Goat	5–19	3–40	25–355
Buffalo	6–28	5–30	5–195

Note: traces of diacetyl were detected in some samples.

Acetaldehyde production by pure cultures of *L. acidophilus* and *S. thermophilus* was maximum at 42°C and 37°C, respectively, and in heated milk at 85°C for 15 min and 65°C for 30 min, respectively, whilst mixed cultures showed more activity in milk steamed for 30 min (Singh, 1983; see also Singh *et al.*, 1982).

Losses of acetaldehyde from yoghurt, after storage for 24 hours, are dependent on the type of milk used for processing, that is, yoghurt made from full fat or whole milk showed little change in acetaldehyde content, while in skimmed milk yoghurt the level decreased (Yu and Nakanishi, 1975a, b). Furthermore, the production of acetaldehyde in yoghurt made from milk of different species can vary. Thus, Gorner *et al.* (1971) observed that acetaldehyde levels, after 3 hour incubation, were highest in yoghurt made from cow's milk, followed by goat's milk and finally sheep's milk; the GLC peak heights of acetaldehyde in these yoghurts were 400, 23 and 2 mm, respectively. The same observation was reported by Abrahamsen *et al.* (1978), where $17.1\,\mu g\,g^{-1}$ of acetaldehyde were present in yoghurt processed from cow's milk, compared to $4.7–5.5\,\mu g\,g^{-1}$ in goat's milk after 3 hours incubation. The behaviour of the yoghurt starter cultures in these different types of milk is not well established, but one of the reasons for the observed changes in metabolism may be that both ewe's and goat's milk contain a substance which blocks the formation of a precursor required by the starter organism for the production of acetaldehyde (see later).

The fate of carbonyl and aroma compounds in yoghurt during storage could be summarised as follows: (a) the levels of acetaldehyde, ethyl acetate and diacetyl in

sheep's milk yoghurt decreased, but the acetone and ethanol contents found in the initial milk showed no change during the fermentation period or storage of the product (Stefanova and Gyosheva, 1985; Georgala *et al*., 1995), (b) acetaldehyde content ($\mu g\,g^{-1}$) decreased in yoghurts made from milk (14.8 to 13.1), milk fortified with SMP (22.8 to 16.5) and UF milk (25.0 to 20.6) (Estevez *et al*., 1988), and (c) the concentration of acetaldehyde decreased in yoghurts stored for 10 days at 4°C or 10°C, whilst the diacetyl and ethanol contents increased (Hruskar *et al*., 1995). However, Kang *et al*. (1988) measured flavour compounds in yoghurt during storage using a dynamic gas-purging headspace technique with a Tenax-GC precolumn or ether extract on a Porapak-Q column, and both methods showed increased acetaldehyde in the product; an observation which was not reported by any other researchers.

Other compounds which could be associated, perhaps indirectly, with flavour enhancement, or act as precursors for the formation of the major aroma compounds in yoghurt, are:

- volatile fatty acids e.g. acetic, propionic, butyric, isovaleric, caproic, caprylic and capric acids (Turcic *et al*., 1969; Dumont and Adda, 1973)
- amino acids e.g. serine, glutamic acid, proline, valine, leucine, isoleucine and tyrosine (Groux, 1976; Grozeva *et al*., 1994)
- products of thermal degradation of milk constituents (i.e. 80–90°C for 15–30 min; Viani and Horman, 1976), for example: (a) from fat (keto acids (acetone, butanone, hexanone), hydroxy acids (ν-valerolactone, δ-caprolactone, δ-caprilactone), and miscellaneous (2-heptanone, 2-nonanone, 2-undecanone, pentane)), (b) from lactose (furfural, furfuryl alcohol, 5-methylfurfural, 2-pentylfuran), (c) from fat and/or lactose (benzyl alcohol, benzyladehyde, methylbenzoate), and (d) from protein (methionine (dimethylsulphide), valine (isobutyraldehyde), or phenylalanine (phenylacetaldehyde) (Haesoo *et al*., 1996))
- *n*-pentaldehyde and 2-heptanone produced by *L. delbrueckii* subsp. *bulgaricus* (Yu and Nakanishi, 1975a, b; Groux and Moinas, 1974).

As mentioned earlier, the formation of acetaldehyde and other aromatic compounds by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in yoghurt takes place during the fermentation, and the final levels are dependent on the presence of specific enzymes which are able to catalyse the formation of carbonyl compounds from the different milk constituents. Lees and Jago (1978a, b) reviewed in detail the role of lactic acid bacteria in terms of flavour production in cultured dairy products, but more is now known of the metabolic mechanisms which lead to the production of flavour and aroma compounds (Zourari *et al*., 1992a; Marshall and Tamime, 1997a). Thus, the possible metabolic pathways of acetaldehyde synthesis are described in the following.

7.2.6.1 Embden–Meyerhof–Parnas pathway

This generates pyruvate (see Fig. 7.1) which in turn is catalysed by α-carboxylase with the formation of acetaldehyde (see also Seneca *et al*., 1950; Lees and Jago, 1966; Keenan and Bills, 1968). Alternatively, the action of pyruvate dehydrogenase on pyruvate results in the formation of acetyl-CoA which can be catalysed/reduced by an aldehyde dehydrogenase to generate acetaldehyde (see also Lees and Jago, 1966,

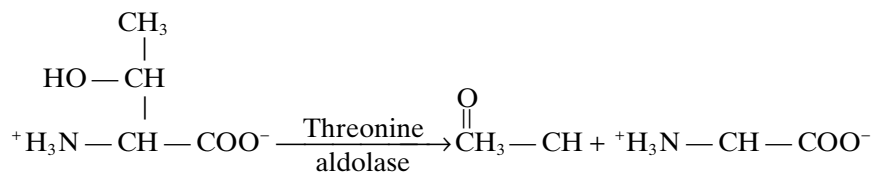
1976a, b, 1977). Lees and Jago (1978a, b) reported aldehyde dehydrogenase activity in four strains each of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, and only two strains of streptococci had alcohol dehydrogenase activity, whilst deoxyriboaldolase was found in one strain of *S. thermophilus*. Nevertheless, Raya *et al.* (1986a) tested two strains of each species of the yoghurt organisms and detected no activities of aldehyde dehydrogenase, phosphoketolase or alcohol dehydrogenase, and only traces of pyruvate decarboxylase activity. Similar observations were reported by Manca de Nadra *et al.* (1988) from the same research laboratory and no deoxyriboaldolase or α-carboxylase activities were detected. Therefore, from these results it is difficult to suggest that acetaldehyde is formed from pyruvate, as the metabolic pathway occurs only rarely in *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. However acetate kinase and phosphotranacetylase were found in some strains (Raya *et al.*, 1986a), whilst aldehyde hydrogenase was found only in *S. thermophilus* (Manca de Nadra *et al.*, 1987, 1988). From these observations it would be difficult to suggest that acetaldehyde could be formed via the hexose monophosphate shunt.

Incidentally, *L. acidophilus* possesses alcohol dehydrogenase activity capable of reducing acetaldehyde so that only a slight yoghurt flavour is found in milk fermented with this culture (Marshall and Cole, 1983). However, diacetyl is produced by *Lactobacillus paracasei* biovar *shirota* from citrate (Marshall, 1987), possibly via the same route of citrate metabolism in *Lactococcus* and *Leuconostoc* species; this *Lactobacillus* is widely used for making yakult (a Japanese fermented milk product).

Benito de Cardenas *et al.* (1991) reported that *L. acidophilus* utilises pyruvate as a carbon source in glucose medium and produces diacetyl; however, diacetyl production is higher in pyruvate medium at 45°C. Greater amounts of acetoin than diacetyl are produced at all temperatures especially 37 and 45°C.

7.2.6.2 Threonine aldolase

This catalyses the cleavage of threonine to acetaldhyde and glycine and both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* possess this enzyme. However, threonine aldolase is more active in the lactobacilli than in the streptococci (Lees and Jago, 1976a, b, 1977). According to Lees and Jago (1978a) the interconversion of threonine to acetaldehyde and glycine is as follows:



Threonine aldolase activity was detected in two strains of *L. delbrueckii* subsp. *bulgaricus*, but not in the two strains of *S. thermophilus* tested (Raya *et al.*, 1986a, b). Other researchers beside Lees and Jago have reported threonine activity in both the yoghurt organisms (Sandine and Elliker, 1970; Wilkins *et al.*, 1986a, b; Marranzini *et al.*, 1989). Streptococcal threonine aldalase activity decreases as the growth temperature increases from 30–42°C, but remains the same in the lactobacilli; since yoghurt is made at ~40–45°C, it is most likely that the acetaldehyde is produced by *L. delbrueckii* subsp. *bulgaricus* (see Zourari *et al.*, 1992a). However, threonine aldolase activity is influenced by glycine level, salts and some divalent cations such

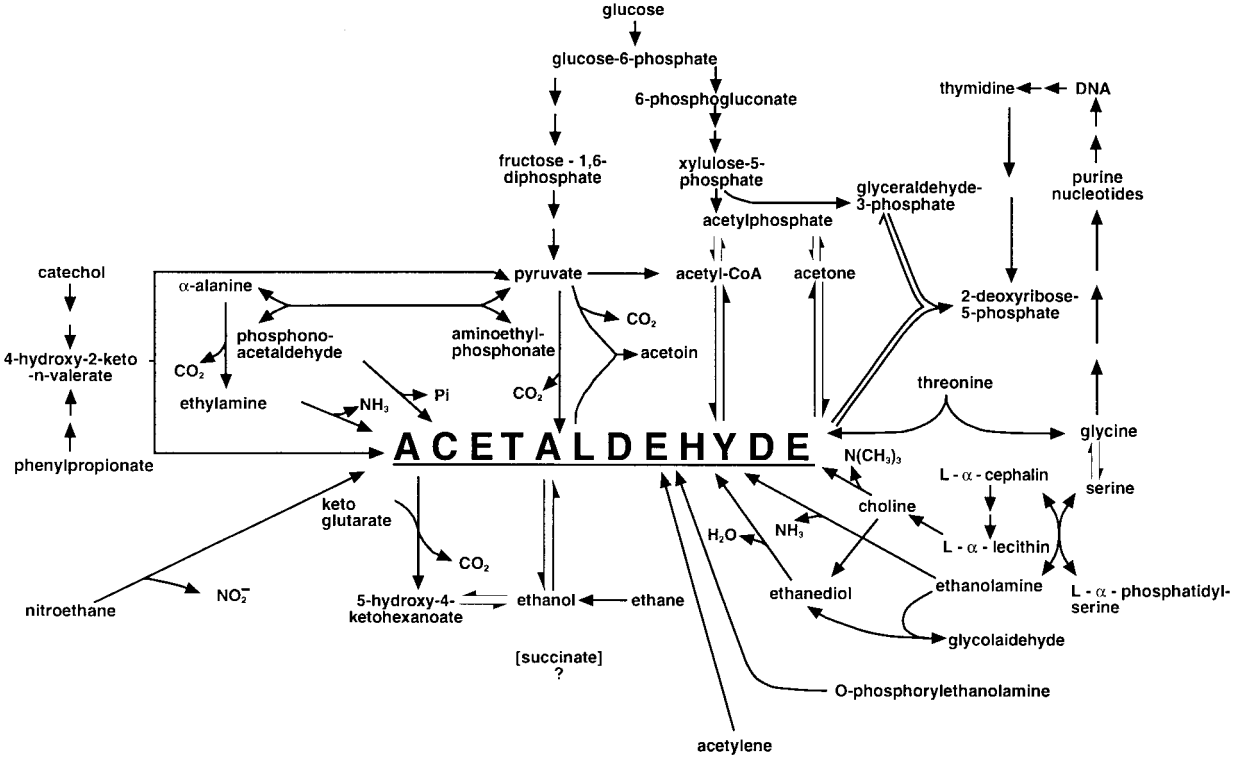


Fig. 7.4 Diagrammatic representation of known reactions involving acetaldehyde

After Lees and Jago (1978a).

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as Cu^{2+}, Zu^{2+}, Fe^{2+} and Co^{2+} (Schmidt *et al*., 1983, 1989; Raya *et al*., 1986a, b; Wilkins *et al*., 1986a; Manca de Nadra *et al*., 1987; Marranzini *et al*., 1989).

Another amino acid, methionine, can also increase the level of acetaldehyde in a growth medium inoculated only with *S. thermophilus* (Shankar, 1977). He observed that by fortifying the growth medium with 100–400 $\mu g\,ml^{-1}$ methionine, the level of acetaldehyde after 20 hour of incubation had increased from 1 $\mu g\,g^{-1}$ in the control to 10 and 14 $\mu g\,g^{-1}$, respectively, in the test media (see also Truffa-Bachi and Cohen, 1968; Rodwell, 1975). Another possible route for the production of acetaldehyde is the cleavage of threonine to glycine, reported by Sandine and Elliker (1970). Flavour production in mutant strains of lactobacilli has been reported by Bednarski and Hammond (1990), whilst glutathione and thiol group production in strains of *S. thermophilus* and *L. helveticus* have been studied by Fernandez and Steele (1993).

7.2.6.3 DNA components

Lees and Jago (1977, 1978a) detected deoxyriboaldolase activity in one of four strains of *S. thermophilus* tested, but this enzyme was not active in *L. delbrueckii* subsp. *bulgaricus* (see also Raya *et al*., 1986a, b). This enzyme, along with thymidine phosphorylase and deoxyribomutase, degrades DNA to 2-deoxyribose-5-phosphate, which is further broken down to acetaldehyde and glyceraldehyde.

It can be observed, therefore, that the production of acetaldehyde by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* may involve a number of different metabolic pathways, and Fig. 7.4 illustrates the possible routes by which acetaldehyde may be formed from carbohydrates, proteins and/or nucleic acids.

7.3 Protein metabolism

Proteolysis in cheesemaking is an important factor in the selection of bacterial strains for starter cultures; however, proteolytic activity of strains used in the manufacture of fermented milks may be of a secondary importance. Nevertheless, although the yoghurt and bio starter cultures are considered to be only weakly proteolytic, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* may, during the fermentation, cause a significant degree of proteolysis and this activity may be important for the following reasons:

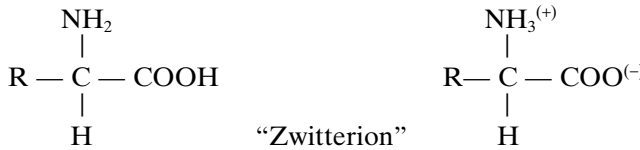
- The enzymatic hydrolysis of milk proteins results in the liberation of peptides of varying sizes and free amino acids, and these possible changes may be involved during the formation of the gel and can affect the physical structure of yoghurt.
- As discussed elsewhere (refer to Chapter 6), the liberation of amino acids into the milk is essential to the growth of *S. thermophilus*.
- Although amino acids and peptides may not contribute directly towards the flavour of yoghurt, they do act as precursors for the multitude of reactions which produce flavour compounds (see Groux, 1976; Viani and Horman, 1976).
- Important nutritional considerations apply through the release of so-called functional peptides (Tomé, 1998).

The range of products released by proteolysis is dependent on two main factors, first, the components of the milk protein fraction and second, the types of proteolytic enzyme that the yoghurt and bio organisms may possess.

7.3.1 Constituent compounds of the milk protein molecule

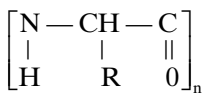
The protein fraction in milk is composed of casein and whey proteins and although the protein molecule is highly complex, it is important in the present context to describe briefly the structure of the protein molecule and show where hydrolysis may occur.

The basic constituents of a protein molecule are compounds known as amino acids. There are about 21 different types of amino acid which have been identified in milk proteins. Their basic structure is shown here



Each amino acid may consist of one or more amino group (NH_3^+) and one or more carboxyl group (COO^-). All the amino acids show asymmetry about the α-carbon atom – where the amino group is next to the carboxyl group – with the exception of glycine where R = H. The nomenclature of the amino acids is similar to that of the carbohydrates, that is, D and L indicate their configuration about the α-carbon atom. Some amino acids are cyclic (e.g. proline which is referred to as an imino acid) but their structure is similar to α-amino acids.

These amino acids are the basic units of the protein molecule and polypeptide chains are built up of sequences of amino acid residues (see Walstra and Jenness, 1984); the structure of the chain is shown here



The buildup of a polypeptide chain results in a loss of water from the amino acids and the bonds between the adjacent units are known as peptide bonds (e.g. —NH.CO—). These polypeptide chains then link together due to the presence of various forces (e.g. hydrogen bonds, covalent and noncovalent bonds) and this aggregation leads to the formation of the protein molecule.

7.3.2 Proteolytic enzymes

These enzymes, as the name suggests, are specific in their action, and their main function is to catalyse the hydrolytic cleavage of the peptide bonds which form the backbone of the protein molecule. The action of the proteolytic enzymes on the peptide bond may be represented as follows:

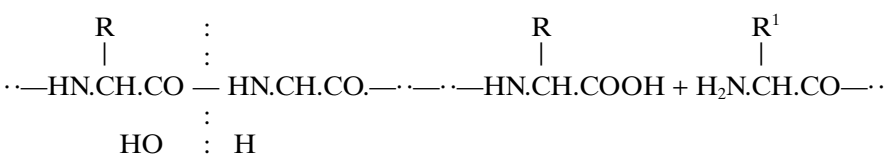


Table 7.4 Enzyme nomenclature of peptidases

Enzyme	Classification and general characteristics
Exopeptidases EC 3.4.11–19 These enzymes act only near the ends of polypeptide chains	Aminopeptidases EC 3.4.11.1–18 *EC 3.4.11.8*[a] now 3.4.19.3 and *EC 3.4.11.11*[a] deleted entry; these enzymes act at a free N-terminus liberating a single amino acid residue Peptidase EC 3.4.13.1–20 Six have been transferred to other EC numbers and two entries[a] deleted; these enzymes catalyse specifically dipeptides. Dipeptidyl-peptidases and tripeptidyl-peptidases EC 3.4.14.1–10 *EC 3.4.14.3*[a] now 3.4.19.1, *EC 3.4.14.7*[a] deleted entry and *EC 3.3.14.8*[a] now 3.4.14.9 & 10; these enzymes act at a free N- terminus liberating a di- or tripeptide. Peptidyl-dipeptidases EC 3.4.15.1–4 *EC 3.4.15.2*[a] now 3.4.19.2 and *EC 3.4.15.3*[a] now 3.4.15.1; these enzymes act at a free C-terminus liberating a dipeptide. Serine-type carboxypeptidases EC 3.4.16.1–4 *EC 3.4.16.3*[a] now 3.4.16.1; these enzymes act at a free C-terminus liberating a single residue. Metallocarboxypeptidases EC 3.4.17.1–17 *EC 3.4.17.5*[a] deleted entry; *EC 3.4.17.7*[a] now 3.4.19.10 and *EC 3.4.17.9*[a] now 3.4.17.4; these enzymes require divalent cations for activity. Cysteine-type carboxypeptidases EC 3.4.18.1 These enzymes act at a free C-terminus liberating a single residue, and require thiol dependence for activity. Omega peptidases EC 3.4.19.1–10 *EC 3.4.19.4*[a] deleted entry; these enzymes remove terminal residues that are substituted, cyclised or linked by isopeptide bonds, i.e. other than those of α-carboxyl or α-amino groups.
Endopeptidases EC 3.4.21–24 & 3.4.99 These enzymes are classified on the basis of catalytic mechanism and specificity	Serine endopeptidases EC 3.4.21.1–74 Ten have been transferred to other EC[a] numbers and eleven deleted entries[a]; these enzymes have an active centre serine of involved in the catalytic process. Cysteine endopeptidases EC 3.4.22.1–35 Ten have been transferred to other EC numbers and two deleted entries[a]; these enzymes have a cystein in the centre. Aspartic endopeptidase EC 3.4.23.1–34 Five have been transferred to other EC numbers and three deleted entries[a]; these enzymes depend on an aspartic acid residue for their catalytic activity. Metalloendopeptidases EC 3.4.24.1–54 Two have been transferred to other EC numbers and four deleted entries[a]; these enzymes use a metal ion (e.g. Zn^{2+}) in the catalytic mechanism. Endopeptidases of unknown catalytic mechanism EC 3.4.99.35–46 Major changes occurred in this section (see Anon., 1992).

[a] Indicate changes that occurred since the last publication of *Enzyme Nomenclature*.

Data compiled from Anon. (1992).

Enzymes acting on peptide bonds are known as peptide hydrolases and to date (1998), a large number of such enzymes have been identified. In the past the name given to an enzyme was derived from the substrate involved, but this approach has created such confusion in the field of enzymology, that the Nomenclature Committee of the International Union of Biochemistry amd Molecular Biology was established to consider a classification of universal application to enzymes and coenzymes. The latest communication of this committee was published (Anon., 1992) and the scheme for classifying and numbering the enzymes is as follows:

- The first number after EC (enzyme classification) indicates to which of the six main classes the enzyme belongs.
- The second figure indicates the subclass.
- The third figure gives the sub-subclass.
- The fourth figure is the serial number of the enzyme in its sub-subclass.

It is not acceptable (Anon., 1992) for the term peptidases to be used as synonymous with peptide hydrolases for the entire group of enzymes that hydrolyse peptide bonds. This is a change from the restriction of peptidases to the enzymes included in the sub-subclasses the exopeptidases and the term proteinase has been replaced by endopeptidases; for consistency, the sub-subclasses of peptidases are recognised as:

- Exopeptidases (EC 3.4.11–19)
- Endopeptidases (EC 3.4.21–24 and EC 3.4.99)

and their overall classification/characteristics is summarised in Table 7.4.

It is probable that this system will be widely adopted in due course, and hence in the present text, the terms endopeptidases and exopeptideses are used in accordance with the new scheme. The hydrolysis of protein to yield amino acids can, therefore, be accomplished in two major stages:

$$\text{Protein} \xrightarrow[\text{Endopeptidases}]{1^{st}\ \text{Stage}} \text{Polypeptides} \xrightarrow[\text{Exopeptidases}]{2^{nd}\ \text{Stage}} \text{Amino acids}$$

7.3.3 Proteolysis by the yoghurt and bio organisms

The data compiled by Tamime and Deeth (1980) on the proteolytic activity of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* indicate that both organisms possess different exopeptidases and peptidases. Since 1980 the proteolytic systems of lactic acid bacteria have been studied in detail using genetic, biochemical and ultrastructural methods. Reviews by Thomas and Pritchard (1987), Kok (1990), Zourari *et al*. (1992a), Pritchard and Coolbear (1993), Vescovo *et al*. (1995), Klaenhammer (1995), Kunji *et al*. (1996) and Law and Haandrikman (1997) describe the properties, regulations and cellular localisation of such enzymes of lactic acid bacteria. However, Bianchi-Salvadori *et al*. (1995) have profiled a wide range of enzymatic activities of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* which were isolated from commercial yoghurts and Italian cheeses; the former organism is considered to have more exopeptidase activity than *L. delbrueckii* subsp. *bulgaricus*, and only limited endopeptidase activity. The ability of *L. delbrueckii* subsp. *bulgaricus* to hydrolyse casein confirms that endopeptidase activity is much higher in

Table 7.5 Proteolysis of individual caseins by different starter cultures

Organism	Sequence of hydrolysis	References
S. thermophilus	β- > α-casein	Shidlovskaya and Dyachenko (1968)
	β- and κ- but not α_{s1}-casein	Desmazeaud and Juge (1976)
	κ- > α_s- and β-casein	Singh and Sharma (1983)
	κ- and α_s- > β-casein	Hegazi (1987)
L. delbrueckii subsp. *bulgaricus*	α_s- > κ- but not β-casein	Ohmiya and Sato (1968, 1969, 1978)
	β- > α_s-casein	Dyachenko and Shidlovskaya (1971)
	κ- > α_s- and β-casein	Chebbi *et al*. (1974)
	κ- and α_s- but not β-casein	Chebbi *et al*. (1977)
	β- > α_s- and κ-casein (whole)	Singh and Ranganthan (1977a, b, 1979)
	α_s- > β- and κ-casein (purified)	
	α_s- and κ- > β-casein	
	β- > α_s- > whole > κ-casein	Shankar and Davies (1978)
	β- > α_s- > κ-casein	Argyle *et al*. (1976), Chandan *et al*. (1982)
	κ- > α_s- and β-casein	Singh and Sharma (1983)
	κ- and α_s- > β-casein	Hegazi (1987)
	β- > α_s- and κ-casein	Moon and Kim (1986, 1990a, b)
	α_s- > κ- and β-casein	Moon *et al.* (1989a, b)
	α_s- and β-casein	Laloi *et al*. (1991), Abraham *et al*. (1993), Gilbert *et al*. (1997)
L. acidophilus	κ- > α_s- and β-casein	Singh and Sharma (1983)
	α_s- and β- but not κ-casein	Hebert *et al*. (1998)
L. helveticus	κ- and α_s- > β-casein	Hegazi (1987)
	α_s- and β- but not κ-casein	Zevaco and Gripon (1988), Yamamot *et al*. (1993, 1994), Martin-Herandez *et al*. (1994)
L. paracasei subsp. *paracasei*	β-casein	Kojic *et al*. (1991), Naes and Nissen-Meyer (1992)

the lactobacilli. This pattern of peptide hydrolysis in the yoghurt organisms provides further evidence of the associative growth relationship which exists between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Thus, the endopeptidase activity of *L. delbrueckii* subsp. *bulgaricus* hydrolyses the casein to yield polypeptides, which in turn are broken down by the exopeptidases of *S. thermophilus* with the liberation of amino acids.

The endopeptidases from *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and bio cultures that are capable of hydrolysing the casein fractions are shown in Table 7.5 (see also Poznanski *et al*., 1965). The pattern of casein catabolism by the yoghurt organisms, which is predominantly attributed to endopeptidase activity, may vary. With the limited data available on casein hydrolysis it is difficult to generalise, but the increased proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* mutant strains (see Table 7.5) developed after exposure to γ-ray radiation, X-ray radiation, UV radiation or chemical mutagens, suggests that differences between ordinary strains may have resulted in the observed variations in casein hydrolysis (see also Dilanian *et al*., 1970, 1971; Krsev, 1976; Singh and Ranganathan, 1974a, b, 1978; Singh *et al*., 1978; Singh and Kaul, 1982a, b). Although mutant strains of *L. delbrueckii* subsp. *bulgaricus* with increased proteolytic activity were not specifically selected for the yoghurt industry, such activity is desired during the early maturation stages of some varieties of Swiss type cheese.

Laloi *et al*. (1991) observed that the endopeptidase present in the cell wall extract of *L. delbrueckii* subsp. *bulgaricus* was active on caseins (see Table 7.5), displayed the same hydrolytic patterns as whole cells, was strongly activated by dithiothreitol and partially inhibited by E-64 (i.e. a specific inhibitor of cysteine endopeptidase); the purified enzyme was not able to hydrolyse di- or tripeptides. However, Oberg *et al*. (1991) used amino acids analysis and the *o*-phthaldialdehyde test to characterise the proteolytic activity of 35 strains of *L. delbrueckii* subsp. *bulgaricus*, and the amino acid profiles provided a cluster analysis to differentiate the strains which was not available from the results of the other test. Furthermore, the caseinolytic activity of endopeptidase from *L. delbrueckii* subsp. *bulgaricus* had the following characteristics: the enzyme was zinc dependent, it degraded intact caseins with a significant preference for β-casein, and the caseinolytic activity increased as the pH was lowered (<5.0) which suggests that the enzyme could be involved in the later stages of the fermentation period (Stefanitsi and Garel, 1997) (see also Stefanitsi *et al*., 1995).

Metalloendopeptidase activity in *S. thermophilus* has been reported by many authors (Sato and Nakashima, 1965; Desmazeaud and Hermier, 1968; Rabier and Desmazeaud, 1973; Desmazeaud, 1974, 1978; Desmazeaud and Zevaco, 1976; El-Soda *et al*., 1978a, b; Shankar and Davies, 1978). More recently, Shahbal *et al*. (1991) reported that the endopeptidase activity of two dairy strains of *S. thermophilus*, CNRZ 385 and 703, was cell wall-associated and not released in the absence of $CaCl_2$, as is the case with *Lactococcus lactis* subsp. *lactis*. Also the high acidification rate of the two strains was correlated with the presence of a 10- and sevenfold increase in endopeptidase activity, respectively, compared with other *S. thermophilus* strains; however, the endopeptidase-negative mutants did not produce higher than average levels of acid.

The cell wall-associated endopeptidase in *L. paracasei* subsp. *paracasei*, *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* has been biochemically characterised and reported by Ezzat *et al.* (1985, 1987), El-Soda *et al*. (1986b, c), Laloi *et al*. (1991)

and Martin-Hernández *et al.* (1994). The proteolytic activity of some of these bacterial species is chromosome linked (El-Soda *et al.*, 1989), and the gene encoding the cell surface endopeptidase from *L. delbrueckii* subsp. *bulgaricus* has been recently sequenced by Gilbert *et al.* (1996); no plasmids have been detected in most of the strains. Furthermore, a comparison of DNA sequences for the cell surface endopeptidases of *L. delbrueckii* subsp. *bulgaricus* and lactococci showed little genetic homology (Gilbert *et al.*, 1996). The endopeptidase, which was purified from *L. delbrueckii* subsp. *bulgaricus*, was a monomer of ~70 kDa, and it was inhibited by EDTA and serine enzymes (Bockelmann *et al.*, 1996). Heating cells of yoghurt lactobacilli at 67–68°C for 15.5–16 s reduced endopeptidase activity, but retained aminopeptidase activity (Lopez-Fandino and Ardö, 1991). Endopeptidases from *L. paracasei* subsp. *paracasei* and *S. thermophilus* showed greatest activity in phosphate buffer followed by tris-HCl, but very low activity in phthalate buffer, whilst similar enzymes from *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* had greatest activity in tris-HCl and lowest in citrate buffer (Akuzawa *et al.*, 1983, 1984). However, when the cell surface caseinolytic activities of *L. paracasei* subsp. *paracasei*, *Lactobacillus delbrueckii* subsp. *lactis* and *L. helveticus* were compared, the characteristics of these endopeptidases of the former organisms were similar; *L. helveticus* displayed two endopeptidases with different cleavage specificities (Gilbert *et al.*, 1997).

The proteolytic system of *L. paracasei* subsp. *paracasei* strains has been investigated (Kojic *et al.*, 1991; Holck and Naes, 1991; Naes and Nissen-Meyer, 1992), and similar PrtP and PrtM genes were identified on the chromosomes; when sequenced, the PrtP gene appeared similar to lactococcal PrtP.

Following the hydrolysis of, for example, the casein in milk, the derived peptides need to be hydrolysed further by the exopeptidases that are present in the yoghurt and bio organisms. Until the 1970s, many authors made reference to exopeptidase activity of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (see the review by Tamime and Deeth, 1980). Currently, the general characteristics of exopeptidases of lactic acid bacteria are given below.

7.3.3.1 Aminopeptidase N (PepN)

In all the organisms studied (see Table 7.6), this enzyme has a molecular weight of ~95 kDa, is a monomeric metallopeptidase, and in most, if not all, it is located intracellularly. The PepN, which was purified from *L. delbrueckii* subsp. *bulgaricus* (Bockelmann *et al.*, 1992), was inhibited completely by 0.1 mM EDTA, and its activity increased by 1 mM Mn^{2+} and 0.1 mM Hg^{2+}; suitable substrates for the assay of enzyme activity were L-Lys-Na and L-Ala-L-Arg-NA. A similar enzyme from *L. helveticus* had a primary sequence PepN identical to the enzymes of *L. delbrueckii* subsp. *lactis* and *Lac. lactis* subsp. *cremoris* (Christensen *et al.*, 1995; Kunji *et al.*, 1996). However, PepN is capable of cleaving N-terminal amino acids, but the enzyme from *L. paracasei* subsp. *paracasei* is only capable of hydrolysing tripeptides containing proline in either the first or second position (Arora and Lee, 1990, 1992; see also Arora *et al.*, 1990).

Characterisation of aminopeptidases N of *L. acidophilus* and other lactobacilli has been reported by El-Soda and Desmazeaud (1982), Ezzat *et al.* (1982, 1986), Hickey *et al.* (1983a, b), Atlan *et al.* (1989), Machuga and Ives (1984) and Khalid *et al.* (1991). Whilst the PepN from *S. thermophilus* was inhibited by $CuCl_2$, $ZnCl_2$ and EDTA, the enzyme showed activity towards *p*-nitroanilide derivatives or di- and

Table 7.6 Some characteristics of exopeptides of selected starter cultures

Enzyme	Micro-organism	Type of Enzyme[a]	Mw[b] (kDa)	Optimum pH activity	References
Aminopeptidase N (PepN)	*L. delbrueckii* subsp. *bulgaricus* B 14	M	95	7.0	Bockelmann *et al.* (1992)
	L. paracasei subsp. *paracasei* LGG	M	87	7.0	Arora and Lee (1992)
	L. helveticus CNRZ 32	NR	97	6.5	Khalid and Marth (1990a, b), Varmanen *et al.* (1994), Christensen *et al.* (1995)
	LHE 511	M	92	7.0	Miyakawa *et al.* (1992)
	ITGL 1	M	97	6.5	Blanc *et al.* (1993)
	S. thermophilus ACA-DC 114	NR	89	6.5	Tsakalidou and Kalantzopoulos (1992)
	CNRZ 302	M	97	7.0	Rul *et al.* (1994), Rul and Monnet (1997)
	NCDO 537	NR	96	NR	Midwinter and Pritchard (1994)
Aminopeptidase C (PepC)	*L. delbrueckii* subsp. *bulgaricus* B 14	T	54	7.0	Wohlrab and Bockelmann (1993)
	L. helveticus CNRZ 32	T	50	NR	Fernández *et al.* (1994), Vesanto *et al.* (1994)
Aminopeptidase X[d] (PepX)	*L. delbrueckii* subsp. *bulgaricus* B 14	S	95	6.5	Bockelmann *et al.* (1991)
	CNRZ 397	S	82	7.0	Atlan *et al.* (1990)
	LBU 47	S	90	6.5	Miyakawa *et al.* (1991)
	L. paracasei subsp. *paracasei* LLG	S	79	8.0	Habibi-Najafi and Lee (1994a)
	L. helveticus CNRZ 32	S	95[c]	7.0	Khalid and Marth (1990b), Kunji *et al.* (1996)
	L. acidophilus	S	95	6.5	Bockelmann *et al.* (1991)
	S. thermophilus	S	165	>6.5	Meyer and Jordi (1987)
Prolinase (PepR)	*L. helveticus* CNRZ 32	NR	35	7.5	Kunji *et al.* (1996)
Proline iminopeptidase	*L. delbrueckii* subsp. *bulgaricus* CNRZ 397	S	3	6.5	Atlan *et al.* (1994), Gilbert *et al.* (1994)
Dipeptidase (PepV)	*L. delbrueckii* subsp. *bulgaricus* B 14	M	51	7.5	Wohlrab and Bockelmann (1992)
	L. helveticus SBT 217 & CNRZ 32	M	50	NR	Tan *et al.* (1995), Kunji *et al.* (1996)
(PepD)	53/7 & CNRZ 32	T	54	6.0	Kunji *et al.* (1996)

[a] M, metallopeptidase; T, thiolpeptidase; S, serine-protease. [b] Molecular weight. [c] Refer to text.
NR Not reported.

tripeptides (Rul *et al*., 1994; Rul and Monnet, 1997); the gene sequence for the enzyme showed high homology with the sequence for PepN isolated from *Lac. lactis* subsp. *cremoris*. A similar enzyme was studied by Tsakalidou and Kalantzopoulos (1992) which was capable of degrading substrates by hydrolysis of N-terminal amino acids and it had very low endopeptidase and no carboxypeptidase activity (see also Kalantzopoulos *et al*., 1990a, b; Tsakalidou *et al*., 1992, 1993).

7.3.3.2 Aminopeptidase C (PepC)

This enzyme is similar to PepN and is capable of removing a broad range of N-terminal residues of peptides; it is a thiol peptidase ~50 kDa. According to Law and Haandrikman (1997), the amino sequence of PepC revealed significant homology with the active site regions of cysteine endopeptidases including papain and mammalian belomycin hydrolase. Recently, Wohlrab and Bockelmann (1992, 1993, 1994) characterised an aminopeptidase from *L. delbrueckii* subsp. *bulgaricus* as similar to the lactococcal PepC; reducing agents such as dithiothreitol and β-mercaptoethanol increased enzyme activity, whilst chelating agents had an inhibitory effect. The site specificity of such enzymes is limited to dipeptides containing N-terminal hydrophobic amino acids, such as Leu-Leu and Ley-Gly (see also Table 7.6).

7.3.3.3 X-prolyl-dipeptidy-laminopeptidase (PepX)

The release of dipeptides from oligopeptides can be accomplished by PepX even when proline is in the penultimate position. Also, PepX is capable of releasing N-terminal prolyl-proline dipeptides from oligopeptides (see the reviews by Mulholland, 1994; Kunji *et al*., 1996; Law and Haandrikman, 1997). The name of this enzyme has, however, been abbreviated to aminopeptidase X and has been extracted and purified from a wide range of lactic acid bacteria (see Table 7.6).

The PepX isolated from *L. delbrueckii* subsp. *bulgaricus* strains and *L. acidophilus* were ~90 kDa, serine-proteases, and were severely inhibited by diisopropyl fluorophosphate (1 mM) and divalent metal ions (1 mM Cu^{2+}, Zn^{2+}, Fe^{2+} or Hg^{2+}) (Miyakawa *et al*., 1991; Bockelmann *et al*., 1991). In mutant strains, PepX was totally deficient and this absence caused a decrease in growth rate, an increase in cell wall endopeptidase activity and a loss of three cell wall proteins (Atlan *et al*., 1990).

The molecular weight of PepX isolated from *L. helveticus* ranged from 72 to 95 kDa, and the spread is possibly due to strain variation or to the method used to calculate the molecular weight (e.g. derived amino acid sequence of cloned gene, by gel filtration or sodium dodecyl sulphate-polyacrylamide gel electophoresis (SDS-PAGE); see also Ardö and Jönsson, 1994; Gatti *et al*., 1997). The same enzyme was found in *L. delbrueckii* subsp. *lactis* and *S. thermophilus* (Meyer and Jordi, 1987), and the molecular weight was 165 kDa in both species; below pH 5, both PepX isolates were unstable and the specificities towards various substrates, including the effect of metals, chelator and other inhibitors, varied with the microbial species.

7.3.3.4 Miscellaneous exopeptidases and endopeptidases

Tripeptidase from *L. delbrueckii* subsp. *bulgaricus* B14 of 85 kDa has been purified and characterised. The enzyme consists of three subunits and a metal-dependent enzyme with an optimum temperature (40°C) and pH (6.0) (Bockelmann *et al*.,

1995, 1997). The characteristics of the prolidase gene (PepQ) and related cryptic gene (OrfZ) from *L. delbrueckii* subsp. *bulgaricus* have been reported by Rantanen and Palva (1997); the properties of enzymes, such as prolinase (PepR) (see also Varmanen *et al*., 1998), proline iminopeptidase and dipeptidases (PepV and PepD), found in lactic lactobacilli are shown in Table 7.6 (see also Habibi-Najafi and Lee, 1994b, 1995; Kim *et al*., 1996).

Little data are available on the proteolytic activity of bio cultures. It could be argued, however, that such microfloras do not grow to any extent during the manufacture of fermented milks and hence the proteolytic activity of the bio starter may be of secondary importance. Nevertheless, Goh *et al.* (1989) reported that, in full fat milk cultured with *B. bifidum* or *L. acidophilus*, soluble nitrogen compounds and free amino acids increased, suggesting that these organisms possess proteolytic enzymes. This view was confirmed by Abu-Taraboush *et al*. (1998) who observed that certain strains of bifidobacteria showed higher proteolytic activity in cultured camel's milk than in cow's milk. The proteolytic activity of *Bifidobacterium longum*, *infantis* and *adolescentis* is attributed to the presence of one aminopeptidase and two dipeptidases in each strain (El-Soda *et al*., 1992; see also Desjardins *et al*., 1990); the properties of aminopeptidase and proline iminopeptidase from *Bifidobacterium breve* have been studied by Cheng and Nagasawa (1985a, b). Nevertheless, the proteolytic activity of the yoghurt organisms appears to be at a maximum under the following conditions:

- Most intense activity is during the log phase.
- The rate of proteolysis decreases during storage or after the stationary phase has been reached.
- The ratio of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the starter culture and the storage period can affect the level of amino acids in yoghurt, and for example, 70 mg 100 g^{-1} is liberated at a ratio of 1:1 after 1 day, followed by 50 mg 100 g^{-1} after 2 days and 41 mg 100 g^{-1} after 5 days. However, the acidity of these yoghurts was rather high, i.e. 1.9 g 100 g^{-1} lactic acid for the 1:1 ratio, and it is possible that the high level of liberated amino acids in the product was associated with the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* which becomes the predominant organism in such an acidic environment (refer later for further discussion).
- In yoghurt (24 hours old) the spectrum of amino acids changes in relation to the ratio of cocci:rods (i.e. at a ratio of 1:1, tryrosine, phenylalanine and leucine formed 56% of the amino acid pool but, at a ratio of 3:1, proline accounted for 71% of the free amino acids).
- The hydrolysis of whey proteins in milk yields lower levels of non-protein nitrogen as the ratio of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus* is decreased.
- Free fatty acids, e.g. capric and, to a lesser degree, oleic, can reduce the proteolytic activity of the starter cultures and can affect the texture of the coagulum.
- Enhanced proteolytic activity in yoghurt is observed during the manufacture of lactose-hydrolysed yoghurt, due perhaps to protease residues present in the β-D-galactosidase preparations (Hemme *et al.*, 1979).
- Milk which was precultured with psychotrophic bacteria prior to the manufacture of yoghurt had enhanced proteolytic activity; however, the product developed unacceptable flavours (see Chapter 2).

Table 7.7 Soluble nitrogenous fractions from milk and milk cultured with the yoghurt micro-organisms

	Dialysable N		Ammonia N		Amino acid N		Urea N		Peptide N	
	$mg\,l^{-1}$	%	$mg\,l^{-1}$	%	$mg\,l^{-1}$	%	$mg\,l^{-1}$	%	$mg\,l^{-1}$	%
Milk	249	4.7	30	0.6	20	0.4	62	1.2	137	2.6
L. delbrueckii subsp. *bulgaricus*										
Av (6)	490	9.3	73	1.4	166	3.1	96	1.8	155	2.9
Range	438–545	8.3–10.3	63–89	1.2–7.9	56–314	1–5.7	51–146	1–2.8	71–270	1.3–5.4
S. thermophilus										
Av (5)	302	5.7	144	2.7	21	0.4	10	0.6	127	2.4
Range	222–406	4.2–7.7	88–190	1.7–3.6	16–26	0.3–0.5	3–30	0.1–0.9	117–197	2.0–3.7

Data compiled from Miller and Kandler (1967a, b). After Tamime and Deeth (1980). Reprinted with permission of *Journal of Food Protection*.

- Bitterness in yoghurt is usually attributed to the production of bitter peptides by the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus*; however, fermentation of the milk at 44°C yields yoghurt which is less likely to be bitter than yoghurt produced at 38°C.

7.3.4 Products of proteolysis

The profile of nitrogenous compounds in yoghurt, compared with milk, changes due to the proteolytic activity of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, both during the fermentation period and, to a lesser degree, during the cold storage of the product. Basically, the change amounts to an increase in the level of soluble nitrogenous compounds, which also includes the liberation of amino acids and the release of peptides from the milk proteins.

7.3.4.1 Soluble nitrogenous compounds

The most comprehensive study in this field was conducted by Miller and Kandler (1967a, b) and a summary of their results is given in Table 7.7. These figures confirm that different strains of yoghurt organisms vary in their proteolytic activity and further, that the amounts of dialysable nitrogen released by *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (490 and 302 $mg\,l^{-1}$, respectively) are compliant with the view that the former organism is more proteolytic than *S. thermophilus*. The same trend can be observed in relation to the amounts of amino acid nitrogen, urea nitrogen and peptide nitrogen (see Table 7.7), but the especial capacity of *S. thermophilus* to increase the level of ammonia nitrogen in cultured milks is due to the ability of the lactic streptococci/lactococci to split urea.

7.3.4.2 Liberation of amino acids

The spectrum of free amino acids in milk and yoghurt (see Table 7.8) is dependent on several variables such as:

- *Type of milk:* milks from different species (cow's, sheep's and goat's) have different contents of amino acids, i.e. ≤10, 3.78 and 20.6 $mg\,100\,ml^{-1}$, respectively, and in addition, goat's milk has, relative to the others, much higher levels of alanine, glycine, glutamic acid, serine and threonine.

Table 7.8 Free amino acid content (mg 100 ml^{-1}) of milk and yoghurt

Amino acid	Cow's		Goat's		Sheep's	
	Milk	Yoghurt	Milk	Yoghurt	Milk	Yoghurt
Alanine	0.16–0.64	1.17–3.80	1.33	3.83	0.56	1.30
Arginine	0.16–0.96	0.70–1.39	0.40	0.67	0.26	0.85
Aspartic acid	0.23–0.52	0.70–1.20	0.22	1.37	0.18	1.75
Glycine	0.30–0.53	0.28–0.45	5.91	6.06	0.15	0.25
Glutamic acid	1.48–3.90	4.80–7.06	3.54	3.78	1.08	4.10
Histidine	0.11	0.80–1.70	0.45	1.28	0.10	0.50
Isoleucine	0.06–0.15	0.15–0.40	0.18	0.43	0.06	0.25
Leucine	0.06–0.26	0.70–1.82	0.21	1.25	0.23	0.45
Lysine	0.22–0.94	0.80–1.11	0.60	2.35	0.19	0.72
Methionine	0.05	0.08–0.20	0.10	0.35	0.05	0.15
Phenylalanine	0.05–0.13	0.17–0.61	0.11	0.35	0.08	0.15
Proline	0.12	5.40–7.05	0.65	4.35	0.11	4.30
Serine	0.08–1.35	1.50–2.90	3.05	3.51	0.20	2.00
Threonine	0.05–0.26	0.24–0.70	3.34	2.80	0.13	0.55
Tryptophan	Tr	0.2	NR	NR	NR	NR
Tyrosine	0.06–0.14	0.18–0.61	0.30	0.60	0.16	0.24
Valine	0.10–0.25	0.90–1.86	0.30	0.50	0.24	0.90
Total	3.29–10.31	18.77–33.06	20.60	33.48	3.78	18.46

Tr; trace. NR; not reported.

Data compiled from Tamime and Deeth (1980).

- *Methods of manufacture:* slightly higher levels of amino acids are obtained when the fermentation is carried out at 42°C for 2–3 hours, rather than at 42°C for 1 hour followed by 5–6 hours at 30–32°C; the total amino acid contents of such yoghurts were 23.6 and 19.4 mg 100 ml^{-1} (Rasic *et al*., 1971a, b; Stojslavljevic *et al*., 1971).
- *Ratio of rods to cocci:* due to the fact that *L. debrueckii* subsp. *bulgaricus* is more proteolytic than *S. thermophilus*, the higher the ratio of rods to cocci in the starter culture, the higher the amino acid content is likely to be in the corresponding yoghurt. Nachev (1970) studied various strains of *L. delbrueckii* subsp. *bulgaricus* and classified them into three groups based on fermentation of sugars and types of amino acid released. The first group (118 strains) was characterised by releasing amino acids (leucine, glutamic acid, asparagine and proline) and an absence in the medium of β-alanine, tryptophan and aminobutyric acid. The second group (six strains) differed in that no glutamic acid was released, while the third group (one strain) was noted for the presence of tryptophan. Profiling of the amino acid content of Finnish fermented milk products has been reported by Kahala *et al*. (1993), who found a high content of proline compared to other amino acids. The glutamic acid content was also high.
- *Conditions during storage:* the temperature of storage of yoghurt can affect the level of free amino acids in the product, i.e. the higher the storage temperature, the greater the increase in free amino acids. Ottogalli *et al.* (1974) stored full and low fat natural yoghurts at 4°C and 20°C for a duration of 60 days and the

increases in the level of amino acids in these yoghurts were (at 4°C) 2.36 and 1.00, and (at 20°C) 7.57 and 14.65 mg 100 ml^{-1}, respectively. However, the same workers observed no increase in the level of amino acids in lemon and orange flavoured yoghurts stored under the same conditions for the same period of time, a difference that was attributed to the presence of natural metabolic inhibitors in the fruit, or the effect of some bacteriocidal agent added to the fruit concentrate, or the high acidity of the fruit preparation.

- *Level of lactic acid:* the amino acid content of yoghurt is dependent on the titratable acidity of the product. According to Luca (1974), yoghurts which contained 1.9 and 1.72–1.73 g 100 g^{-1} lactic acid had total amino acid contents of 70 and 41–50 mg 100 g^{-1}, respectively. Incidentally, the figure of 70 mg 100 g^{-1} in yoghurt is the highest level reported in the literature and it could be argued that such acidic yoghurt could be the result of prolonged incubation, and hence the amino acid content reflects directly the extent of the metabolic activity of the starter culture.

The final amino acid content of yoghurt made from cow's milk may range from 18.7 to 33 mg 100 ml^{-1} (see Table 7.8) and it is probable that the acidities of these yoghurts were 1.0–1.4 g 100 g^{-1} lactic acid. It is important, of course, that the total amino acid content of yoghurt reflects a balance between proteolysis and assimilation by the bacteria. Some amino acids, such as glutamic acid, proline and, to a lesser degree, alanine and serine, are presumably not required by the yoghurt organisms and thus accumulate in larger quantities in the product than the remaining amino acids which are utilised by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* during growth and/or fermentation.

7.3.4.3 Release of peptides

As mentioned earlier, some of the proteolytic enzymes which the yoghurt bacteria possess release peptides into the product. Some work carried out on this aspect of the fermentation has been reported from Bulgaria by Tanev and Zivkova (1977) and in this study, the behaviour of the short chain peptides in Bulgarian yoghurt during cold storage was monitored. The technique of peptide mapping, which included high voltage electrophoresis and finger printing by descending paper chromatography and differential staining of the peptides, was neatly demonstrated on both milk and yoghurt stored at 4°C for 1, 2, 3 and 65 days. The size and composition of these short chain peptides was not given, but the distribution of these peptides in yoghurt has been reported.

Kahala *et al.* (1993) reporting on the rate of proteolysis and peptide profiles of Finnish fermented milks (e.g. Bulgarian yoghurt, natural/plain yoghurt, biokefir and acidophilus milk) found that the rate of proteolysis increased during the storage period and the highest rate of proteolysis was found in fresh biokefir and after storage compared with other fermented milk products. However, the peptide profiles for Bulgarian and natural yoghurts were similar. The identified fractions were: Leu, Tyr, Phe, α_{s1}-casein 1–14, β-casein 47–57, β-casein 166–175 and β-casein 176–188 (see also Kyriakidis *et al.*, 1993; Weimer *et al.*, 1989); factors affecting the formation of amines in the growth medium by *L. delbrueckii* subsp. *bulgaricus* have been reported by Chander *et al.* (1989).

7.4 Lipid/fat metabolism

7.4.1 Introduction

Acyl glycerols constitute 96–98% of the total milk lipids/fats and the remaining fraction consists of phospholipids, sterols, fat-soluble vitamins (A, D, E and K), fatty acids, waxes and squalene. The lipids are found in the following phases of the milk: the fat globules, the membranes of the fat globules and the milk serum. The proportions of these fractions can vary in relation to such factors as species of mammal, breed, stage of lactation and type of feed (Walstra and Jenness, 1984; Weihrauch, 1988; Fox, 1991, 1994). The acyl glycerols present in milk are formed by the esterification of the alcohol radicals of the glycerol with one, two or three fatty acids residues to yield mono-, di- or triacylglycerides (triglycerides), respectively. Therefore, in broad terms, the enzymatic hydrolysis of milk lipids takes place at the ester linkages, eventually yielding free fatty acids and glycerol. The enzymes are known as triacylglycerol lipases EC 3.1.1.3 (Anon., 1992) and their mode of action may be specific to certain bonds on the glycerol molecules, that is, similar to the action of the peptidases (see Section 7.3.2). A simplified sequence of lipids hydrolysis is as follows:

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Di-} \xrightarrow{\text{Lipase}} \text{Mono-} \xrightarrow{\text{Lipase}} \text{Fatty acids + glycerol}$$

The triacylglycerol lipase enzymes in yoghurt may originate from the starter culture or from microbial contaminants that survived the heat treatment of the milk. Incidentally, the lipases, which occur naturally in milk, are inactivated at ordinary pasteurisation temperatures (Deeth and Fitz-Gerald, 1976). Therefore, any reduction in the percentage of fat, or increase in the level of fatty acids (free or esterified), or increase in the content of volatile fatty acids in yoghurt can be attributed to lipid metabolism by micro-organisms, including *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. However, before evaluating the role of the different lipases reported to be present in the latter organisms, it is pertinent to look at some factors which can affect the degree of lipolysis.

7.4.1.1 Fat content of yoghurt

The fat content ($g\,100\,g^{-1}$) of yoghurt differs from one country to another according to the existing or proposed standards for the chemical composition of the product, or alternatively in relation to the types of yoghurt produced. There are four broad categories of yoghurt and related products:

- fat free or <1%
- >1% and <3%
- >3% and <4%
- >4.5% and 10%

and the degree of lipolysis is likely to be greater in yoghurts with high fat contents.

7.4.1.2 Homogenisation

The process is carried out on the milk base and is widely practised in the yoghurt industry for two main reasons, first, to reduce the size of the fat globules and thus prevent "creaming" or fat separation in the milk during incubation, and second, to

Table 7.9 Triacylglycerol lipase activities of the yoghurt starter cultures

Enzyme	Substrate	*S. thermophilus*	*L. delbrueckii* subsp. *bulgaricus*
Tributyrase	Tributyrin	+++[a]	++
Trioleinase	Soy-milk and olive oil		
Glycerol ester hydrolase	Milk fat	++	
Enterases	Tween 40 and 60 and α-napthyl acetate or butyrate	+	+++
	Triacetin		Tr
Tricaproinase	Tricaproin		+

[a] Owing to different enzyme assay procedures employed, the enzyme activities are expressed as high (+++), medium (++) or low (+).

Tr = Trace.

Data compiled from Morichi *et al.* (1968), Otterholm *et al.* (1968), Angeles and Marth (1971), Formisano *et al.* (1972, 1973, 1974) and Umanskii *et al.* (1974).

improve the viscosity and texture of yoghurt. However, the extent of lipolysis in homogenised milk is much greater than in non-homogenised milk, due, in large measure, to the destruction of the protective layer of the fat globule, that is, the fat globule membrane (Mulder and Walstra, 1974).

Although the hydrolysis of fat by the yoghurt starter cultures occurs only to a limited degree, it may still be enough to contribute towards the flavour of the product. In fact, only Formisano *et al.* (1974) reported any appreciable loss of lipids, namely a decrease of 3.4% in the fat in yoghurt stored for 21 days at 4°C. This observation was not noted by other workers.

However, several authors in the 1960s and 1970s detected lipase activity in *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, and a list of these enzymes is shown in Table 7.9; the nomenclature of the enzymes is based on the substrate being hydrolysed, rather than on the systematic approach suggested by Anon. (1992). Nevertheless, all these triacylglycerol lipases in the yoghurt bacteria are reported to be located in the cytoplasm, since after cell disruption, very little activity is associated with the cell membrane (see also DeMoraes and Chandan, 1982); the fatty acid composition of dairy starter cultures has been reported by Rezanka *et al.* (1983) and Chand *et al.* (1992). Recently, Kalantzopoulos *et al.* (1990a, b) reported esterase activity in both yoghurt organisms and these enzymes were extracted from either the cell wall or the interior of the cell. The percentage of esterase activity was also high in *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Bianchi-Salvadori *et al.*, 1995).

The characterisation of esterase activities of lactobacilli species has been reported by El-Soda *et al.* (1986a, b) and Khalid *et al.* (1990) and could briefly be summarised as follows:

- Enzyme activities using nitrophenyl derivatives of fatty acids were recorded as positive towards derivatives up to 50°C.
- *P*-nitrophenyl derivatives were hydrolysed faster than the *O*-nitrophenyl derivatives.

Table 7.10 Changes in the free fatty acid contents of yoghurt made with milks from different mammals

Fatty acids	Cow	Sheep	Goat
Caproic	–	I	–
Caprylic	I	I	D
Capric	–	–	–
Lauric	I	I	D
Myristic	I	I	D
C-15	–	D	–
Palmitic	I	D	I
Palmitoleic	–	–	–
Stearic	D	D	–
Oleic	D	D	–
Linoleic	–	–	I

(I) Increase } by more than 1% compared with milk. (–) Signifies
(D) Decrease } no change.

Data compiled from Rasic and Vucurovic (1973) and Rasic *et al.* (1973).

- *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* strains had lower esterase activities than *L. acidophilus* and *L. delbreuckii* subsp. *lactis*.
- The enzymes activities were optimum at pH ~7.0, and at temperatures in the range between 40 and 50°C.
- Freezing of cells, growth medium (e.g. MRS, sterile skimmed milk or whey-based medium) and stage of growth can influence esterase activities in *Lactobacillus* species (see also El-Sawah *et al*. 1995; Nadathur *et al*., 1996).

7.4.2 Changes in the level of free and esterified fatty acids

The free and esterified fatty acids of yoghurts made from cow's, sheep's and goat's milk were studied by Rasic and Vucurovic (1973) and Rasic *et al.* (1973), and the changes which occurred are summarised in Table 7.10. From such data, it seems that the increase (or decrease) in the level of free fatty acids in the different types of yoghurt is inconsistent, and this variation probably reflects a difference in behaviour of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in cow's, sheep's and goat's milk (see also Boccignone *et al*., 1983, 1985).

In another investigation from another laboratory (Formisano *et al.*, 1974), the reported change in the free fatty acids in yoghurt was somewhat simplified, in that there was a liberation of long chain fatty acids into the product and the final pattern did not change significantly during cold storage. However, fermentation of full fat milk with *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* or *L. acidophilus* resulted in different effects on milk lipids, and according to Rao and Reddy (1984) the changes were as follows:

- Significant increase in saturated fatty acids and oleic acid.
- A concomitant decrease in linoleic and linolenic acids in the glyceride fraction.

Table 7.11 Changes in volatile fatty acids (VFA) in whole and skimmed milk fermented at 37° for different durations with yoghurt organisms

Fatty acids		Milk[a]	*S. thermophilus*		*L. delbrueckii* subsp. *bulgaricus*		Mixed	
			24 hs	72 hs	24 hs	72 hs	24 hs	72 hs
Total VFA ($mg\,100\,g^{-1}$)	W	3.20	6.05	6.26	4.90	4.19	6.88	7.55
	S	2.97	5.89	6.32	4.88	3.79	6.80	7.20
C2	W	0.21	0.55	1.26	0.51	0.45	0.57	0.48
	S	0.20	1.95	1.36	0.45	0.37	0.12	0.20
C3	W	Tr	Tr	Tr	0.05	0.03	0.22	0.11
	S	–	0.05	0.05	0.03	0.03	Tr	Tr
i-C4	W	0.03	0.03	0.05	0.05	0.04	0.13	0.14
	S	0.03	0.04	0.61	0.05	0.05	0.03	0.06
n-C4	W	0.39	0.74	0.94	1.21	0.97	1.05	1.44
	S	0.38	0.50	0.96	1.20	0.90	0.66	1.08
i-C5	W	0.05	0.21	0.21	0.14	0.10	0.15	0.06
	S	0.03	0.13	0.18	0.11	0.09	0.07	0.17
n-C5	W	–	–	–	–	–		
	S	–	Tr	Tr	–	–		
n-C6	W	1.09	1.73	1.24	1.24	1.05	1.56	2.57
	S	1.13	1.72	1.35	1.25	1.07	2.40	2.04
C8	W	0.97	1.44	0.99	0.74	0.53	1.78	1.64
	S	0.96	1.30	1.18	0.87	0.56	2.26	2.36
C10	W	1.21	1.59	1.30	0.91	1.10	2.65	2.22
	S	1.10	1.81	1.74	1.06	0.68	3.11	2.92

[a] W: whole milk; S: skimmed milk.

Tr: Trace. (–): not detected. Empty space signifies test was not determined.

Data compiled from Yu *et al*. (1974) and Yu and Nakanishi (1975a–c).

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- The increase in free fatty acids was moderate, but these were significant increases in stearic and oleic acids.
- The monoglyceride fraction disappeared completely upon fermentation.
- The changes in cholesterol content were not significant.
- A significant correlation ($r = 0.711$) was found between acid degree value and the level of free fatty acids.

7.4.3 Changes in the level of volatile fatty acids

During the manufacture and storage of yoghurt, there is an appreciable increase in the total level of volatile fatty acids in the product. Data on the release of these fatty acids by single strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and by mixed cultures have been reported by many investigators, and of the two organisms, the *Lactobacillus* produces more of these acids than *S. thermophilus*. The increase in the level of volatile fatty acids in yoghurt is dependent on several variables, such as the strains of starter bacteria, type of milk (i.e. cow's, buffalo's or

goat's), duration and temperature of incubation, temperature of heat treatment of the milk and/or the age of yoghurt (Dutta *et al.*, 1971a, b, 1973; Singh *et al.*, 1980). However, a slight decrease in volatile fatty acids was observed in the presence of low concentrations of citric acids in milk (Dutta *et al.*, 1972).

Yu *et al.* (1974, 1985) and Yu and Nakanishi (1975a–c) have reported in detail on the levels of certain fatty acids in whole and skimmed milk cultured with yoghurt starter bacteria. Their data are shown in Table 7.11, and it can be observed that after 24 hours of incubation at 37°C, only a small degree of lipolysis has been exhibited by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. It could be argued, however, that the origin of volatile fatty acids in fermented milks, and in particular in those based on skimmed milk, may not be the result of lipid metabolism by the yoghurt organisms, but may arise from the breakdown of other milk constituents (e.g. the amino acid pool), as suggested by Nakai and Elliot (1965); in the course of oxidative deamination and decarboxylation, the amino acid is split into its corresponding volatile fatty acid. The lipid constituents of skimmed and full fat vita (i.e. Bulgarian fermented milk made with *L. delbrueckii* subsp. *bulgaricus*) have been reported by Ilinova and Naumova (1984).

Howover, Morichi *et al.* (1968) have pointed out that the presence of "true detected esterases" in the lactic acid bacteria (e.g. *L. delbrueckii* subsp. *bulgaricus*) is difficult to verify, since some of the proteolytic enzymes and other factors in milk may exhibit esterase activity. Consequently, it is safe to assume that the detected esterase activity of the yoghurt bacteria (see Table 7.9) is directly related to the action of proteolytic enzymes rather than lipases. Such a conclusion is in accord with the higher production of volatile fatty acids by *L. delbrueckii* subsp. *bulgaricus*, that is it is probably due to endopeptidases and/or exopeptidases rather than lipases.

7.5 Vitamin metabolism

7.5.1 General background

Milk contains both fat- and water-soluble vitamins. Table 7.12 (see also Chapter 9) indicates the levels of these vitamins in different milks (full fat and skimmed) and in the corresponding yoghurts (see also Ashoor *et al.*, 1983, 1985; Scott and Bishop, 1986; Rao and Shahani, 1987; Laukkanen *et al.*, 1988; Delgado Zamarreno *et al.*, 1996). The content of these vitamins changes during manufacture for the following reasons.

7.5.1.1 Decrease

- An excess of dissolved oxygen and/or a moderate heat treatment of milk can reduce significantly its vitamin content and the most susceptible vitamins are C, B_6, B_{12} and folic acid (see Chapter 2, Table 2.20).
- Excessive heat treatments of the milk, e.g. boiling for 5 min, cause even greater losses of the above vitamins; for example, vitamin B_{12} is reduced by 1.78 $\mu g l^{-1}$ (Rasic and Panic, 1963).
- The yoghurt starter bacteria utilise some of the vitamins present in milk during the fermentation period to meet their growth requirements. This factor contributes, to some extent, to a reduction in the nutritional properties of the product. However, the quantities consumed are dependent on the rate of

Table 7.12 Vitamin contents of different milks and yoghurts[a]

Vitamin/ units	Cow		Cow		Goat		Sheep		Soya[f]	
	SS milk[b]	Yoghurt[c]	Milk[d]	Yoghurt[e]	Milk	Yoghurt	Milk	Yoghurt	Milk	Yoghurt
I. ($\mu g\,100\,g^{-1}$)										
Retinol	1	8	52	28	44	N	88	86	0	23
Carotene	Tr	5	21	21	Tr	Tr	Tr	Tr	Tr	Tr
Vitamin D	Tr	0.01	0.03	0.04	0.11	N	0.18	0.24	0	0
Vitamin B_{12}	0.4	0.2	0.4	0.2	0.1	Tr	0.6	0.2	0	0
Folate	5	17	6	18	1	7	5	3	19	N
Biotin	1.9	2.9	1.9	2.6	3	0.5	2.5	N	N	N
II. ($mg\,100\,g^{-1}$)										
Vitamin E	Tr	0.01	0.09	0.05	0.03	0.03	0.11	0.73	0.74	1.49
Thiamin	0.04	0.05	0.03	0.06	0.04	0.04	0.08	0.05	0.06	N
Riboflavin	0.17	0.25	0.17	0.27	0.13	0.17	0.32	0.33	0.27	N
Niacin	0.09	0.15	0.08	0.18	0.31	0.27	0.41	0.23	0.11	N
Tryptophan 60	0.78	1.20	0.75	1.33	0.73	0.83	1.27	1.03	0.52	0.88
Vitamin B_6	0.06	0.09	0.06	0.10	0.06	0.06	0.08	0.08	0.07	N
Pantothenate	0.32	0.45	0.35	0.50	0.41	0.23	0.45	N	N	N
Vitamin C	1	1	1	1	1	1	1	Tr	0	0

[a] Plain or natural yoghurt. [b] Semi-skimmed milk (1.6 g fat $100\,g^{-1}$). [c] Low fat yoghurt (0.8 g fat $100\,g^{-1}$). [d] Full fat milk (3.9 g fat $100\,g^{-1}$). [e] Full fat yoghurt (3.0 g fat $100\,g^{-1}$). [f] The product may be fortified with retinol and vitamin E.

Tr: Trace.

N: nutrient is present in significant quantities, but there is no reliable information on the amount.

Data compiled from Holland *et al.* (1989).

Table 7.13 Effect of incubation temperature upon vitamin synthesis in yoghurt

Vitamin ($\mu g\,100\,g^{-1}$)	Milk + 2% SMP	Incubation temperature (°C) for 3 hours			
		37	40	42	45
Folic acid	0.37	3.74	4.04	4.32	3.94
Niacin	120	126	130	142	136

After Reddy *et al.* (1976).

inoculation, the strain of yoghurt starter and the conditions of fermentation (Shahani *et al.*, 1974; Friend *et al.*, 1983).

- Some vitamins decrease during the storage of yoghurt at 4°C, i.e. vitamin B_{12} (Rasic and Panic, 1963; Cerna *et al.*, 1973). Reddy *et al.* (1976) observed losses of folic acid and vitamin B_{12} of 28.6 and 59.9%, respectively, during the storage of yoghurt at 5°C for 16 days. The same workers also observed a decrease in the biotin, niacin and pantothenic acid contents. They attributed these losses to the combined effect of microbial catabolism during the incubation period and chemical decomposition of these vitamins during cold storage. This latter aspect was confirmed in yoghurt made by the direct acidification method rather than by microbial fermentation (see also Scott and Bishop, 1986; Saidi and Warthesen, 1993; Sharma *et al.*, 1996).
- A folic acid producing strain of *S. thermophilus* increased the folic acid content of yoghurt after 3.5 hours, and then the level decreased rapidly; this indicates that as the *L. delbrueckii* subsp. *bulgaricus* starts to grow, it utilises the vitamin produced by *S. thermophilus* (Kaneko *et al.*, 1987). Also, the same workers observed that a wide range of lactobacilli utilised vitamin B_{12} when grown in reconstituted skimmed milk (see also Wachol-Drewek and Roczniak, 1982; Rao *et al.*, 1984).
- EPS-producing yoghurt starter organisms decreased the thiamin and biotin contents in the product, whilst non-EPS cultures increased the contents of biotin, folic acid and riboflavin (Erzinkyan *et al.*, 1987).
- *L. acidophilus* and *B. bifidum* utilised the folic acid present in milk (Drewek and Czarnocka-Roczniakowa, 1983).
- A long incubation of yoghurt (i.e. incubation at 30°C for 14–16 hours) decreased the synthesis of folic acid, but increased the content of thiamin and nicotinic acid in the product (Kneifel *et al.*, 1989).

7.5.1.2 Increase

Vitamins which increase during the actual manufacture of yoghurt are niacin and folic acid, because they are actively synthesised by the starter cultures. According to Reddy *et al.* (1976), the increases in folic acid and niacin in yoghurt (made from whole milk fortified with 2% SMP and incubated for 3 hours at 42°C) amounted to 3.95 and $22\,\mu g\,100\,g^{-1}$, respectively (see also Table 7.13); losses in storage (see above) may exceed these gains in due course. Although there is a general agreement in the literature that vitamin B_{12} decreases during yoghurt production, Mitic *et al.* (1974), Shahani *et al.* (1974) and Kilara and Shahani (1976, 1978) found that some species of *Lactobacillus* and strains of yoghurt starter culture synthesise vitamin B_{12}.

The reported folic acid contents in commercial yoghurt may range between 3.7 and 24.5 μg 100 g^{-1} (Kaneko *et al*., 1987; Hoppner and Lampi, 1990; Wigertz *et al*., 1997), and mutant strains of *S. thermophilus* increased the folic acid content in skimmed milk to 38.1 μg 100 g^{-1} (Kaneko *et al*., 1987). Furthermore, as mentioned elsewhere, non-EPS yoghurt cultures increased the biotin, folic acid and riboflavin contents in the fermented product (Erzinkyan *et al*., 1987). However, enhanced synthesis of vitamins in yoghurt can be achieved by using different combinations of starter cultures. Examples are the inclusion of *Propionibacterium* spp. in the yoghurt starter cultures increased the folic acid content in the product by 43% (Wachol-Drewek and Roczniak, 1983), yoghurt made with added *Saccharomyces cerevisiae* and preservatives had higher riboflavin and niacin contents during storage (Durga *et al*., 1986), and a mixed culture of bifidobacteria, *L. delbrueckii* subsp. *bulgaricus* and kefir grains at a ratio of 1:0.5:0.5 increased the thiamin and riboflavin contents in the product by 27% and 18%, respectively (Khamagacheva *et al*., 1988).

In the early 1990s, Austrian researchers studied a total of 47 commercially available starter cultures (e.g. yoghurt, bio-cultures and kefir), and the results (see below) suggested different patterns of synthesis and utilisation of water-soluble vitamins in fermented milks (Kneifel *et al*., 1989, 1991).

In view of the existing evidence (see also Deeth and Tamime, 1981), it is safe to conclude that *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* synthesise niacin and folic acid and, to a lesser degree, vitamin B_6 during the production of yoghurt. Evidence of vitamin synthesis by *Bifidobacterium* species has been reported by Ballongue (1998) and there is evidence of biotin synthesis by different bifidobacterial strains (Noda *et al*., 1994). Thus, taking into consideration that both the yoghurt micro-organisms and some bio starter cultures are capable of synthesising certain water-soluble vitamins, it is of some interest to consider the possible metabolic pathway(s) involved in the synthesis of these vitamins.

7.5.2 Biosynthesis of folic acid

The folic acid group (or folates) is a generic name given to around ten different compounds which share a basic structural unit connected to conjugates of different numbers of glutamic acid residues. These folates are, therefore, made from carbon, hydrogen, nitrogen and oxygen atoms, and their formulae range from $C_{15}H_{12}N_6O_4$ to $C_{49}H_{61}N_{13}O_{24}$. Thus, some or all of these compounds are active as folacin and a typical structure of one such compound (i.e. pteroylglutamic acid [*p*-(2-amino-4-oxodihydropteridyl-6)-methyl-aminobenzoyl-L-glutamic acid]), which may be synthesised by the bacteria is shown in Fig. 7.5.

Many organisms require folacin as a growth factor. It functions as a coenzyme in many different biochemical reactions (i.e. as an activator and carrier of carbon units during oxidation) and it participates in the metabolism of purines, pyrimidines and some amino acids. However, the synthetic pathways of folic acid in *S. thermophillus* and *L. delbrueckii* subsp. *bulgaricus* are not well established, and Lentner (1984, 1986) suggested that the synthesis of this compound in animals, plants and micro-organisms probably involves the biochemical reactions shown in Fig. 7.6.

It is worthwhile reporting that folate-binding proteins might be involved in folate absorption in the human intestine and that their concentration is important (Wigertz *et al*., 1997), but since the milk base is heated to temperatures ≥90°C during

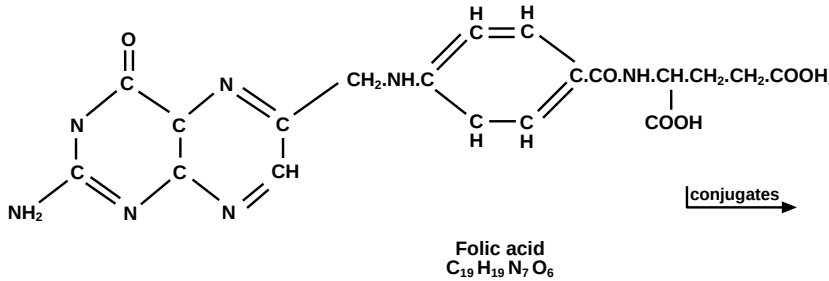

Fig. 7.5 Possible structure formation of folic acid by yoghurt micro-organisms

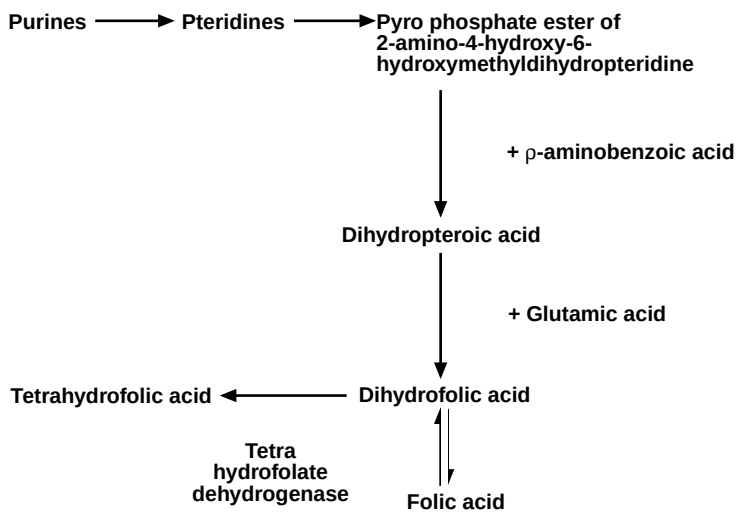

Fig. 7.6 Probable biochemical reactions during the synthesis of folic acid by micro-organisms

After Lentner (1984, 1986).

manufacture, yoghurt contains significantly lower concentrations of folate-binding proteins compared with other dairy products.

7.5.3 Biosynthesis of niacin

Niacin activity is exhibited by nicotinic acid and nicotinamide. The former compound constitutes part of the structure of two important coenzymes, that is, NAD and nicotinamide adenine dinucleotide phosphate (NADP). These two coenzymes are composed of adenylic acid and nicotinamide ribotide linked through their phosphate groups (see Fig. 7.7); however, NADP contains an additional phosphate group (Stanier *et al.*, 1987). As NAD and/or NADP are essential for many oxidative/reductive biochemical reactions, the niacin synthesised by *S. thermophilus* and *L.*

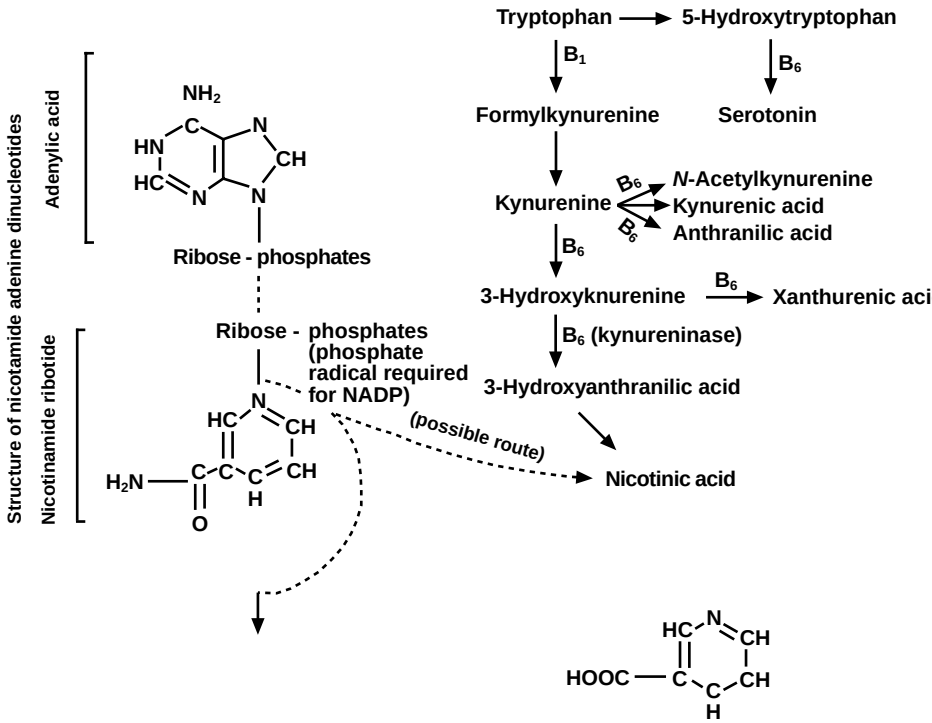

Fig. 7.7 Some possible schemes for the biosynthesis of niacin by yoghurt micro-organisms
Adapted from Lentner (1984, 1986) and Stanier *et al.* (1987).

delbrueckii subsp. *bulgaricus* may originate from the nicotinamide fraction arising during the formation of NAD and/or NADP. The biosynthesis of these nucleotides basically involves the following steps: first, the synthesis of a sugar moiety (possibly derived from the available milk sugar(s)) and second, the synthesis of the pyrimidine or purine base. Alternatively, after this formation of NAD and/or NADP, the nicotinamide fraction could be released as a result of the degradation of these nucleotides, but whether nicotinic acid could be derived from the released nicotinamide must be subject to further investigation.

However, nicotinic acid is derived by a few bacteria from the metabolism or breakdown of tryptophan, a pathway which is dependent on the availability of certain vitamins (e.g. thiamine, riboflavin and vitamin B_6), to activate the required enzymes (Lentner, 1984, 1986). As *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* utilise these vitamins and tryptophan does not accumulate during yoghurt production, it is possible that these organisms use the vitamins for the synthesis of niacin. In view of the limited information in this field, Fig. 7.7 can do no more than illustrate some possible schemes for the synthesis of niacin by the yoghurt microflora.

7.5.4 Biosynthesis of vitamin B_6

The activity of vitamin B_6 is exhibited equally by pyridoxine, pyridoxal and pyridoxamine. The basic structure of these compounds is similar in that it consists

Table 7.14 Levels of water-soluble vitamins in fermented milks

Vitamin	Yoghurt ($n = 30$)	Bio-yoghurt ($n = 14$)	Kefir ($n = 3$)
Units (mg 10 ml^{-1})			
Thiamin	0.034	0.030	0.011
Riboflavin	0.121	0.135	0.145
Pyrodoxine	0.023	0.015	0.011
Nicotinic acid	0.088	0.109	0.076
Units (μg 100 ml^{-1})			
Cobalamin	0.19	0.23	0.27
Folic acid	3.22	3.62	1.38

After Kneifel et al. (1989, 1991).

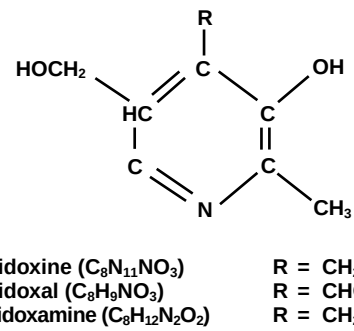

Fig. 7.8 Basic structure of vitamin B_6 including the different radical components

of a pyridine ring, but they differ in respect of the radical components (see Fig. 7.8).

According to Lentner (1984, 1986), no information is available on the biosynthesis of the pyridine ring in micro-organisms, plants or animals; however, the different forms of vitamin B_6 are interconvertible by micro-organisms in accordance with the scheme illustrated in Fig. 7.9. In view of the limited knowledge of the synthesis of vitamin B_6 in general, it is difficult to suggest any possible metabolic pathway by which *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* might synthesise this vitamin.

7.6 Miscellaneous changes

The biological activity of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and bio starter cultures during the manufacture of yoghurt and related products is highly complex. Current scientific work has elucidated some general information about the metabolic pathways employed by these organisms. Nevertheless, numerous changes do occur in the milk and some of the additional minor changes in the milk constituents are: (a) a reduction in the level of citric acid, (b) the content of hippuric

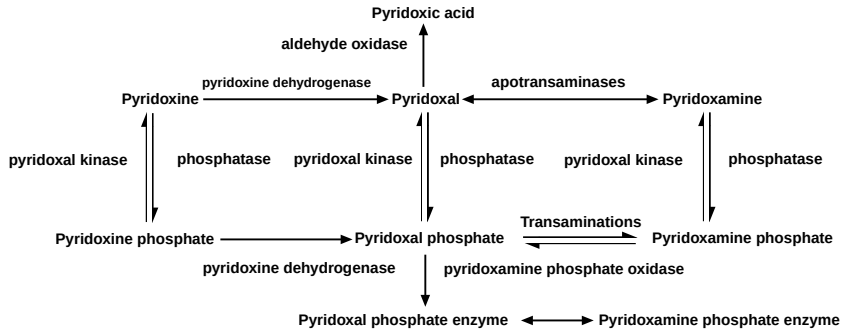

Fig. 7.9 Scheme to illustrate that pyridoxine, pyridoxal, pyridoxamine and their phosphates are interconvertible by micro-organisms

After Lentner (1984, 1986).

acid is lost altogether, and (c) the levels of acetic and succinic acids are increased, especially when bio starter cultures are used to ferment the milk. Other changes that may occur involve the following:

- Uracyl-4-carboxylic acid – this compound is better known as orotic acid or orotate anion ($pk_a = 2.4$). It is metabolised by the yoghurt starter cultures, most probably by *L. delbrueckii* subsp. *bulgaricus* and its content in milk is reduced by up to 50% (i.e. from 8.3 to 3.4–4.2 mg 100 ml^{-1}), during the manufacture of yoghurt (Okonkwo and Kinsella, 1969a; see also Lavanchy and Steiger, 1984; Haggerty *et al*., 1984; Prakash and Sharma, 1986; Navder *et al*., 1990; Saidi and Warthesen, 1989). However, orotic acid possesses some significant therapeutic properties, since it plays an important role in the biosynthesis of nucleic acids. Furthermore, according to Larson and Hegarty (1979), the level of orotic acid in cultured dairy products is dependent on the degree of fermentation and the amount of soluble whey solids in the product (see also Okonkwo and Kinsella, 1969b). Suzuki *et al*. (1986) have reported that pyrimidine biosynthesis from orotic acid may be negatively regulated by the intracellular level of purine nucleotides and *L. delbrueckii* subsp. *bulgaricus* could not grow in milk depleted of orotate; this indicates that pyrimidine synthesis in this micro-organism is very low.
- Metal ions – little is known about the utilisation of minor nutrients, such as metal ions, by lactic acid bacteria. Boyaval (1989) has reviewed the available information on the transport and importance of metal ions. For example: (a) the inhibition of certain exopeptidases of *S. thermophilus* and *L. delbrueckii* subsp. *lactis* by chemicals can be nullified by Co^{2+}, Zn^{2+} or Mn^{2+}, (b) the presence of Mn^{2+} and Mg^{2+} in the growth medium stimulated the growth of *S. thermophilus* and *L. acidophilus*, (c) Fe^{2+} stimulated the growth of *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus*, and (d) *S. thermophilus* required Ca^{2+} for growth, whilst for *L. acidophilus*, Ca^{2+} caused morphological changes (i.e. from filamentous to bacilloid) and the transitioned cells were more freeze resistant.
- 7 α-dehydroxylase activity on bile acids – strains of *Lactobacillus* spp., *Bifidobacterium* spp. and *S. thermophilus* test negative for this enzyme, which suggests that

the intake of these starter cultures is safe because their presence in the human intestine does not produce secondary bile acids that can promote colon cancer (Takahashi and Morotomi, 1994).

- Angiotensin-I-converting enzymes (ACE) – these enzymes tend to release exopeptidases that are associated with the renin–angiotensin system which regulates peripheral blood pressure (Meisel *et al*., 1997). The inhibitory activity of these enzymes has been found to be low in yoghurt, but high in cheeses.
- Enzymatic activities – some enzymatic activities of lactic acid bacteria which might be of interest in the present review are: (a) *S. thermophilus* was the best producer of superoxide dismatase compared with six other lactic starter cultures (Hosono *et al*., 1991), (b) glutamic acid uptake by *S. thermophilus* was energy dependent and NaCl strongly inhibited the uptake (Bracquart *et al*., 1989), and (c) a rapid screening method of the yoghurt microflora for restriction endonuclease activity was reported by Poch and Somkuti (1998).
- Immunostimulating agent – this component contained *N*-acetyl-muramyl peptides which were derived from *L. delbrueckii* subsp. *bulgaricus* (Link and Pahud, 1991); the method of processing was patented and the immunostimulating agent could be used during the manufacture of fermented milk products to promote an immune response against Gram-negative bacteria in the intestine.
- Health benefits – the presence of other metabolites, for example β-galactosidase (Kilara and Shahani, 1976; Rao and Dutta, 1977, 1978), and various antitumour and antimicrobial agents (Reddy *et al*., 1973a; Pulusani *et al*., 1979; Rao and Pulusani, 1981) must not be forgotten, for such agents might be of medical and therapeutic value to humans. However, for an update regarding the health benefits of fermented milks including the production of bacteriocin by the dairy lactic acid bacteria, the reader should refer to Chapters 5 (Section 5.10), 6 and 9.

7.7 References

ABO-ELNAGA, I.G. and HEGAZI, F.Z. (1981) *Nahrung*, **25**, 883.

ABOU-DONIA, S.A., ISMAIL, A.A., EL-SODA, M.A., MASHALY, R. and EL-SHAFY, H.K. (1984) *Indian Journal of Dairy Science*, **37**, 172.

ABRAHAM, A.G., DE ANTONI, G.L. and ANON, M.C. (1993) *Journal of Dairy Science*, **76**, 1498.

ABRAHAMSEN, R.K., SVENSEN, A. and TUFTO, G.N. (1978) *XX International Dairy Congress*, **E**, 828.

ABU-TARABOUSH, H.M., AL-DAGAL, M.M. and AL-ROYLI, M.A. (1998) *Journal of Dairy Science*, **81**, 354.

ADAMS, R.M., YOAST, S., MAINZER, S.E., MOON, K., DALOMBELLA, A.L., ESTELL, D.A., POWER, S.D. and SCHMIDT, B.K. (1994) *Journal of Biological Chemistry*, **269**, 5666.

ADDA, J. (1986) In *Developments in Food Flavours*, Ed. by Birch, G.G. and Lindley, M.G. Elsevier Applied Science, London, pp. 151–172.

AKUZAWA, R., ITO, O. and YOKOYAMA, K. (1983) *Japanese Journal of Zootechnical Science*, **54**, 685.

AKUZAWA, R., ITO, O. and YOKOYAMA, K. (1984) *Dairy Science Abstracts*, **46**, 134.

ALEKSIEVA, P., GIRGINOVA, T. and KONDRATENKO, M. (1981) *Dairy Science Abstracts*, **43**, 381.

ÁLVAREZ, J.A., GELPÍ, J.L., JOHNSEN, K., BERNARD, N., DELCOUR, J., CLARKE, A.R., HOLBROOK, J.J. and CORTÉS, A. (1997) *European Journal of Biochemistry*, **244**, 203.

AMOROSO, M.J. and MANCA DE NADRA, M.C. (1991) *Microbiologie, Aliments, Nutrition*, **9**, 171.

AMOROSO, M.J., MANCA DE NADRA, M.C. and OLIVER, G. (1988) *Milchwissenschaft*, **43**, 626.

AMOROSO, M.J., MANCA DE NADRA, M.C. and OLIVER, G. (1989) *Lait*, **69**, 519.

AMOROSO, M.J., MANCA DE NADRA, M.C. and OLIVER, G. (1992) *World Journal of Microbiology and Biotechnology*, **8**, 50.

ANDALOUSSI, S.A., TALBAOUI, H., MARCZAK, R. and BONALY, R. (1995) *Applied Microbiology and Biotechnology*, **43**, 995.

ANGELES, A.G. and MARTH, E.H. (1971) *Journal of Milk and Food Technology*, **34**, 69.

ANON. (1992) *Enzyme Nomenclature*, The International Union of Biochemistry and Molecular Biology, Academic Press, San Diego, pp. 371–421.

ARDÖ, Y. and JÖNSSON, L. (1994) *Journal of Dairy Research*, **61**, 573.
ARGYLE, P.J., MATHISON, G.E. and CHANDAN, R.C. (1976) *Journal of Applied Bacteriology*, **41**, 175.
ARIGA, H., URASHIMA, T., MICHIHATA, E., ITO, M., MORIZONO, N., KIMURA, T. and TAKAHASHI, S. (1992) *Journal of Food Science*, **57**, 625.
ARIHARA, K. and LUCHANSKY, J.B. (1995) In *Food Biotechnology-Microorganism*, Ed. by Hui, Y.H. and Khachatourians, G.C. VCH Publishers, New York, pp. 609–643.
ARORA, G. and LEE, B.H. (1990) *Journal of Dairy Science*, **73**, 274.
ARORA, G. and LEE, B.H. (1992) *Journal of Dairy Science*, **75**, 700.
ARORA, G., LEE, B.H. and LAMOUREUX, M. (1990) *Journal of Dairy Science*, **73**, 264.
ASHOOR, S.H., SEPERICH, G.J., MONTE, W.C. and WELTY, J. (1983) *Journal of Food Science*, **48**, 92.
ASHOOR, S.H., KNOX, M.J., OLSEN, J.R. and DEGER, D.A. (1985) *Journal of Association of Official Analytical Chemists*, **68**, 693.
ASHOUR, M., EL-ZAYAT, A.I., RABIE, A.H. and GOUDA, A. (1985) *Egyptian Journal of Food Science*, **13**, 137.
ATLAN, D., LALOI, P. and PORTALIER, R. (1989) *Applied and Environmental Microbiology*, **55**, 1717.
ATLAN, D., LALOI, P. and PORTALIER, R. (1990) *Applied and Environmental Microbiology*, **56**, 2174.
ATLAN, D., GILBERT, C., BLANC, B. and PROTALIER, R. (1994) *Microbiology*, **140**, 527.
BAISYA, R.K. and BOSE, A.N. (1975) *Indian Journal of Dairy Science*, **28**, 179.
BALLONGUE, J. (1998) In *Lactic Acid Bacteria*, 2nd Edition, Ed. by Salminen, S. and von Wright, A., Marcel Dekker, New York, pp. 519–587.
BEDNARSKI, W. and HAMMOND, E.G. (1990) *Journal of Dairy Science*, **73**, 1450.
BENATEYA, A., BRACQUART, P. and LINDEN, G. (1991) *Canadian Journal of Microbiology*, **37**, 136.
BENITO DE CARDENAS, I.L., GERUTTI DE GUGLIELMONE, G., LEDESMA, O. and OLIVER, G. (1989) *Microbiologie, Aliments, Nutrition*, **7**, 383.
BENITO DE CARDENAS, I.L., PERAL DE PORTILLO, M.C., ALBIZZATTI DE RIVADENEIRA, M.C. and OLIVER, G. (1991) *Microbiologie, Aliments, Nutrition*, **9**, 369.
BERKELEY, R.C.W., GOODAY, C.W. and ELLWOOD, D.C. (Eds.) (1979) In *Microbial Polysaccharides and Polysaccharases*, Academic Press, London.
BERNARD, N., JOHNSEN, K., FERAIN, T., GARMYN, D., HOLS, P., HOLBROOK, J.J. and DELCOUR, J. (1994) *European Journal of Biochemistry*, **224**, 439.
BERNARD, N., JOHNSEN, K., HOLBROOK, J.J. and DELCOUR, J. (1995) *Biochemical and Biophysical Research Communications*, **208**, 895.
BERNARD, N., JOHNSEN, K., GELPÍ, J.L., ÁLVAREZ, J.A., FERANI, T., GARMYN, D., HOLS, P., CORTÉS, A., CLARKE, A.R., HOLBROOK, J.J. and DELCOUR, J. (1997) *European Journal of Biochemistry*, **244**, 213.
BESHKOVA, D., SIMOVA, E., FRENGOVA, G. and SIMOVA, Z. (1998) *Journal of Industrial Microbiology & Biotechnology*, **20**, 180.
BIANCHI-SALVADORI, B., CAMASCHELLA, P. and CISLAGHI, S. (1995) *International Journal of Food Microbiology*, **27**, 253.
BLANC, B., LALOI, P., ATLAN, D., GILBERT, C. and PORTALIER, R. (1993) *Journal of General Microbiology*, **139**, 1441.
BLUMENTHAL, A. and HELBLING, J. (1974) *Dairy Science Abstracts*, **36**, 331.
BOCCIGNONE, M., BRIGIDI, R. and SARRA, C. (1983) *Dairy Science Abstracts*, **45**, 351.
BOCCIGNONE, M., BRIGIDI, R. and SARRA, C. (1985) *Dairy Science Abstracts*, **47**, 190.
BOCKELMANN, W., FOBKER, M. and TEUBER, M. (1991) *International Dairy Journal*, **1**, 51.
BOCKELMANN, W., SHULZ, Y. and TEUBER, M. (1992) *International Dairy Journal*, **2**, 95.
BOCKELMANN, W., BEUCK, H.P., LICK, S. and HELLER, K. (1995) *International Dairy Journal*, **5**, 493.
BOCKELMANN, W., HOPPE-SYLER, T. and HELLER, K.J. (1996) *International Dairy Journal*, **6**, 1167.
BOCKELMANN, W., GOLLAN, V. and HELLER, K.J. (1997) *Milchwissenschaft*, **52**, 500.
BOTTAZZI, V. (1967) *Dairy Science Abstracts*, **29**, 156.
BOTTAZZI, V. and BIANCHI, F. (1986) *Scienza e Tecnica Lattiero – Casearia*, **37**, 297.
BOTTAZZI, V. and DELLAGLIO, F. (1967) *Journal of Dairy Research*, **34**, 109.
BOTTAZZI, V. and VESCOVO, M. (1969) *Netherlands Milk and Dairy Journal*, **23**, 71.
BOUZAR, F., CERNING, J. and DESMAZEAUD, M. (1996) *Journal of Dairy Science*, **79**, 205.
BOUZAR, F., CERNING, J. and DESMAZEAUD, M. (1997) *Journal of Dairy Science*, **80**, 2310.
BOYAVAL, P. (1989) *Lait*, **69**, 87.
BRACQUART, P., LE DEAUT, J.-Y. and LINDEN, G. (1989) *Journal of Dairy Research*, **56**, 107.
BRANNY, P., DE LA TORRE, F. and GAREL, J.-R. (1996) *Journal of Bacteriology*, **178**, 4727.
BRANNY, P., DE LA TORRE, F. and GAREL, J.-R. (1998) *Microbiology – Reading*, **144**, 905.
LE BRAS, G., DEVILLE-BONNE, D. and GAREL, J.-R. (1991) *European Journal of Biochemistry*, **198**, 683.
CERNA, J., PICKOVA, J. and BLATTNA, J. (1973) *Dairy Science Abstracts*, **35**, 413.
CERNING, J. (1990) *FEMS Microbiology Reviews*, **87**, 113.
CERNING, J. (1994) In *Bactéries Lactique*, Vol. 1, Ed. by de Rossarlaud, H. and Laquet, F.M., Lorica, Uriage, pp. 309–329.
CERNING, J. (1995) *Lait*, **75**, 463.
CERNING, J., BOUILLANNE, C., DESMAZEAUD, M.J. and LANDON, M. (1986) *Biotechnology Letters*, **8**, 625.
CERNING, J., BOUILLANNE, C., DESMAZEAUD, M.J. and LANDON, M. (1988) *Biotechnology Letters*, **10**, 255.

CERNING, J., BOUILLANNE, C., LANDON, M. and DESMAZEAUD, M.J. (1990) *Sciences des Aliments*, **10**, 443.
CERNING, J., BOUILLANNE, C., LANDON, M. and DESMAZEAUD, M.J. (1992) *Journal of Dairy Science*, **75**, 692.
CESCA, B., MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A. and OLIVER, G. (1984) *Folia Microbiologia*, **29**, 288.
CHAND, R., MALAN, C.P. and SRINIVASAN, R.A. (1992) *Journal of Dairying, Foods and Home Sciences*, **11**, 31.
CHANDAN, R.C., ARGYLE, P.S. and MATHISON, G.E. (1982) *Journal of Dairy Science*, **65**, 1408.
CHANDER, H., BATISH, V.K., BABU, S. and SINGH, R.S. (1989) *Journal of Food Science*, **54**, 940.
CHANG, S.K. and HASSAN, H.M. (1997) *Applied and Environmental Microbiology*, **63**, 3722.
CHANG, B.-S. and MAHONEY, R.R. (1989a) *Journal of Dairy Research*, **56**, 117.
CHANG, B.-S. and MAHONEY, R.R. (1989b) *Journal of Dairy Research*, **56**, 785.
CHANG, B.-S. and MAHONEY, R.R. (1994) *Biotechnology and Applied Biochemistry*, **19**, 169.
CHEBBI, N.B., CHANDER, H. and RANGANATHAN, B. (1974) *Journal of General and Applied Microbiology*, **20**, 149.
CHEBBI, N.B., CHANDER, H. and RANGANATHAN, B. (1977) *Acta Microbiologica Polonica*, **9**, 281.
CHENG, C.-C. and NAGASAWA, T. (1985a) *Japanese Journal of Zootechnical Science*, **56**, 257.
CHENG, C.-C. and NAGASAWA, T. (1985b) *Japanese Journal of Zootechnical Science*, **56**, 484.
CHRISTENSEN, J.E., LIN, D., PALVA, A. and STEELE, J.L. (1995) *Gene*, **155**, 89.
COGAN, T.M. (1995) *Journal of Applied Bacteriology (Symposium Supplement)*, **79**, 49S.
COGAN, T.M. and HILL, C. (1993) In *Cheese: Chemistry, Physics and Microbiology*, Vol. 1, 2nd Edition, Ed. by Fox, P.F., Chapman & Hall, London, pp. 193–255.
COLLINS, M.A. and THOMPSON, J.K. (1992) *Journal of Applied Bacteriology*, **73**, 237.
DAVID, S., STEVENS, H., VAN RIEL, M., SIMONS, M.G. and DE VOS, W.M. (1992) *Journal of Bacteriology*, **174**, 4475.
DEETH, H.C. and FITZ-GERALD, C.H. (1976) *Australian Journal of Dairy Technology*, **31**, 53.
DEETH, H.C. and TAMIME, A.Y. (1981) *Journal of Food Protection*, **44**, 78.
DELCOUR, J., BERNARD, N., GARMYN, D., FERAIN, T. and HOLS, P. (1993) *Lait*, **73**, 127.
DELGADO ZAMARRENO, M.M., SANCHEZ PEREZ, A., SANCHES RODRIGUEZ, M., GOMEZ PEREZ, M.C. and HERNANDEZ MENDEZ, J. (1996) *Talanta*, **43**, 1555.
DEMORAES, J. and CHANDAN, R.C. (1982) *Journal of Food Science*, **47**, 1579.
DESJARDINS, M.-L., ROY, D. and GOULET, J. (1990) *Journal of Dairy Science*, **73**, 299.
DESMAZEAUD, M.J. (1974) *Biochimie*, **56**, 1173.
DESMAZEAUD, M.J. (1978) *XX International Dairy Congress*, **E**, 469.
DESMAZEAUD, M.J. and HERMIER, J.H. (1968) *Annales de Biologie Animale Biochimie et Biophysique*, **8**, 565.
DESMAZEAUD, M.J. and JUGE, M. (1976) *Lait*, **56**, 241.
DESMAZEAUD, M.J. and ZEVACO, C. (1976) *Annales de Biologie Animale Biochimie et Biophysique*, **16**, 851.
DILANIAN, Z., MARKARIAN, K. and SARKISYAN, R. (1970) *VIII International Dairy Congress*, **IE**, 122.
DILANIAN, Z. KH., ARUTYUNYAN, R.K., MARKARIAN, K.V. and AKOPYAN, A.A. (1971) *Dairy Science Abstracts*, **33**, 222.
DILLS, S.S., APPERSON, A., SCHMIDT, M.R. and SAIER, J.R., M.H. (1980) *Microbiological Reviews*, **44**, 385.
DOCO, T., FOURNET, B., CARCANO, D., RAMOS, P., LOONES, A., PIOT, J.M. and GUILLOCHON, D. (1989) European Patent Application, EP 0 331 564 A2.
DOCO, T., WIERUSZESKI, J.-M., FOURNET, B., CARCANO, D., RAMOS, P. and LOONES, A. (1990) *Carbohydrate Research*, **198**, 313.
DOCO, T., CARCANO, D., RAMOS, P., LOONES, A. and FOURNET, B. (1991) *Journal of Dairy Research*, **58**, 147.
DREWEK, Z. and CZARNOCKA-ROCZNIAKOWA, B. (1983) *Dairy Science Abstracts*, **45**, 762.
DUMONT, J.P. and ADDA, J. (1973) *Lait*, **53**, 12.
DURGA, C.L., SHARADA, D. and SASTRY, P.M. (1986) *Indian Journal of Dairy Science*, **39**, 404.
DUTTA, S.M., KUILA, R.K., RANGANATHAN, B. and LAXMINARAYANA, H. (1971a) *Indian Journal of Diary Science*, **24**, 107.
DUTTA, S.M., KUILA, R.K., RANGANATHAN, B. and LAXMINARAYANA, H. (1971b) *Milchwissenschaft*, **26**, 158.
DUTTA, S.M., KUILA, R.K., SABHARWAL, V.B. and RANGANATHAN, B. (1972) *Journal of Food Science and Technology – India*, **9**, 207.
DUTTA, S.M., KUILA, R.K. and RANGANATHAN, B. (1973) *Milchwissenschaft*, **28**, 231.
DYACHENKO, P.F. (1971) In *Chemistry of Milk*, Ministry of Meat and Milk Industry, Tallinn, U.S.S.R.
DYACHENKO, P.F. and SHIDLOVSKAYA, V.V. (1971) *Dairy Science Abstracts*, **33**, 56.
EL-SAWAH, M.M.A., SHERIEF, A.A. and BAYOUMY, S.M. (1995) *Antonie van Leeuwenhoek*, **67**, 357.
EL-SODA, M.A. and DESMAZEAUD, M.J. (1982) *Canadian Journal of Microbiology*, **28**, 1181.
EL-SODA, M.A., DESMAZEAUD, M.J. and BERGÉRE, J.L. (1978a) *XX International Dairy Congress*, **E**, 475.
EL-SODA, M.A., DESMAZEAUD, M.J. and BERGÉRE, J.L. (1978b) *Journal of Dairy Research*, **45**, 445.
EL-SODA, M., KORAYEM, M. and EZZAT, N. (1986a) *Milchwissenschaft*, **41**, 353.
EL-SODA, M.A., DESMAZEAUD, M.J., LE BARS, D. and ZEVACO, C. (1986b) *Journal of Food Protection*, **49**, 361.
EL-SODA, M., ABD-EL-WAHAB, H., EZZAT, N., DESMAZEAUD, M.J. and ISMAIL, A.A. (1986c) *Lait*, **66**, 431.
EL-SODA, M., LUCAS, S. and NOVEL, G. (1989) *Egyptian Journal of Dairy Science*, **17**, 201.
EL-SODA, M., MACEDO, A. and OLSON, N.F. (1992) *Milchwissenschaft*, **47**, 87.

ERZINKYAN, L.A., AKOPOVA, A.B., TSIBUL'SKAYA, M.I. and POMORTSEVA, N.V. (1987) *Dairy Science Abstracts*, **49**, 58.
ESCALANTE, A., WACHER-RODARTE, G., GARCIA-GARIBAY, M. and FARRES, A. (1998) *Journal of Applied Microbiology*, **84**, 108.
ESTEVEZ, C.R., GOICOECHEA, A. and JIMENEZ PEREZ, S. (1988) *Dairy Science Abstracts*, **50**, 661.
EZZAT, N., EL-SODA, M., DESMAZEAUD, M.J. and ISMAIL, A. (1982) *Milchwissenschaft*, **37**, 666.
EZZAT, N., EL-SODA, M., BOUILLANNE, C., ZEVACO, C. and BLANCHARD, P. (1985) *Milchwissenschaft*, **40**, 140.
EZZAT, N., EL-SODA, M., DESMAZEAUD, M.J. and ISMAIL, A.A. (1986) *Lait*, **66**, 445.
EZZAT, N., ZEVACO, C., EL-SODA, M. and GRIPON, J.C. (1987) *Milchwissenschaft*, **42**, 95.
FARROW, J.A.E. (1980) *Journal of Applied Bacteriology*, **49**, 493.
FERNANDEZ, L. and STEELE, J.L. (1993) *Journal of Dairy Science*, **76**, 1233.
FERNANDEZ, L., BHOWMIK, T. and STEELE, J.L. (1994) *Applied and Environmental Microbiology*, **60**, 333.
FERNANDEZ-GARCIA, E. and MCGREGOR, J.U. (1994) *Journal of Dairy Science*, **77**, 2934.
FORMISANO, M., PERCUOCO, G. and PERCUOCO, S. (1972) *Dairy Science Abstracts*, **34**, 324.
FORMISANO, M., PERCUOCO, G. and PERCUOCO, S. (1973) *Dairy Science Abstracts*, **35**, 137.
FORMISANO, M., COPPOLA, S., PERCUOCO, G., PERCUOCO, S., ZOINA, A., GERMANO, S. and CAPRIGLIONE, I. (1974) *Annali di Microbiologia ed Enzimologia*, **24**, 281.
FOX, P.F. (Ed.) (1991) In *Food Enzymology*, Vol. 1, Elsevier Science Publishers, London.
FOX, P.F. (Ed.) (1994) In *Advanced Dairy Chemistry: Lipids*, Vol. 2, 2nd Edition, Chapman & Hall, London.
FRIEND, B.A., FIEDLER, J.M. and SHAHANI, K.M. (1983) *Milchwissenschaft*, **38**, 133.
GALESLOOT, TH.E. and HASSING, F. (1966) *Dairy Science Abstracts*, **28**, 184.
GAMAR, L., BLONDEAU, K. and SIMONET, J.M. (1997) *Journal of Applied Microbiology*, **83**, 281.
GANCEL, F. and NOVEL, G. (1994a) *Journal of Dairy Science*, **77**, 685.
GANCEL, F. and NOVEL, G. (1994b) *Journal of Dairy Science*, **77**, 689.
GANCEL, F., NOVEL, G., CARCANO, D., LOONES, A. and RAMOS, P. (1989) French Patent Application, FR 2 632 968 A1.
GARCIA-GARIBAY, M. and MARSHALL, V.M.E. (1991) *Journal of Applied Bacteriology*, **70**, 325.
GARMAN, J., COOLBEAR, T. and SMART, J. (1996) *Applied Microbiology and Biotechnology*, **46**, 22.
GARVIE, E.I. (1978) *Journal of Dairy Research*, **45**, 515.
GARVIE, E.I. (1980) *Microbiological Reviews*, **44**, 106.
GASSEM, M.A., SCHMIDT, K.A. and FRANK, J.F. (1995) *Cultured Dairy Products Journal*, **30**(3), 18.
GASSEM, M.A., SCHMIDT, K.A. and FRANK, J.F. (1997a) *Journal of Food Science*, **62**, 171.
GASSEM, M.A., SIMS, K.A. and FRANK, J.F. (1997b) *Lebensmittel-Wissenschaft und Technologie*, **30**, 273.
GASSER, F. (1970) *Journal of General Microbiology*, **62**, 223.
GASSER, F. and GASSER, C. (1971) *Journal of Bacteriology*, **106**, 113.
GATTI, M., FORNASARI, E. and NEVIANI, E. (1997) *Letters in Applied Microbiology*, **25**, 345.
GEORGALA, A.I.K., TSAKALIDOU, E., KANDARAKIS, I. and KALANTZOPOULOS, G. (1995) *Lait*, **75**, 271.
GERMOND, J.E., HOTTINGER, H., MIGNOT, O., MOLLET, B. and TSUDA, K. (1995) European Patent Application, EP 0 638 642 A1.
GILBERT, C., ATLAN, D., BLANC, B. and PORTALIER, R. (1994) *Microbiology*, **140**, 537.
GILBERT, C., ATLAN, D., BLANC, B., PORTALIER, R., GERMOND, J.E., LAPIERRE, L. and MOLLET, B. (1996) *Journal of Bacteriology*, **178**, 3059.
GILBERT, C., BLANC, B., FROT-COUTAZ, J., PORTALIER, R. and ATLAN, D. (1997) *Journal of Dairy Research*, **64**, 561.
GIRAFFA, G. (1994) *Industrie Alimentari*, **33**, 295.
GIRAFFA, G. and BERGÈRE, J.-L. (1987) *Lait*, **67**, 285.
GOH, J.S., KWON, I.K, AHN, J.K. and YOON, Y.H. (1989) *Dairy Science Abstracts*, **51**, 273.
GORNER, F., PALO, V. and BERTAN, M. (1968) *Milchwissenschaft*, **23**, 94.
GORNER, F., PALO, V. and BERTAN, M. (1971) *Dairy Science Abstracts*, **33**, 800.
GREENBERG, N.A. and MAHONEY, R.R. (1982) *Journal of Food Science*, **47**, 1824.
GREENBERG, N.A. and MAHONEY, R.R. (1984) *Food Chemistry*, **15**, 307.
GREENBERG, N.A., WILDER, T. and MAHONEY, R.R. (1985) *Journal of Dairy Research*, **52**, 439.
GROBBEN, G.J., SIKKEMA, J., SMITH, M.R. and DE BONT, J.A.M. (1995) *Journal of Applied Bacteriology*, **79**, 103.
GROBBEN, G.J., SMITH, M.R., SIKKEMA, J. and DE BONT, J.A.M. (1996) *Applied Microbiology and Biotechnology*, **46**, 279.
GROBBEN, G.J., VAN CASTEREN, W.H.M., SCHOLS, H.A., OOSTERVELD, A., SALA, G., SMITH, M.R., SIKKEMA, J. and DE BONT, J.A.M. (1997) *Applied Microbial and Biotechnology*, **48**, 516.
GROBBEN, G.J., CHIN-JOE, I., KITZEN, V.A., BOELS, I.C., SIKKEMA, J. SMITH, M.R. and DE BONT, J.A.M. (1998) *Applied and Environmental Microbiology*, **64**, 1333.
GROUX, M. (1976) In *Nestlé Research News 1974/75,* Ed. by Boella, C. Nestlé Product Technical Assistance, Lausanne, pp. 50–52.
GROUX, M. and MOINAS, M. (1974) *XIX International Dairy Congress*, **IE**, 293.
GROZEVA, L., TCHORBANOV, B. and GYOSHEVA, B. (1994) *Journal of Dairy Research*, **61**, 581.
GRUTER, M., LEEFLANG, B.R., KUIPER, J., KAMERLING, J.P. and VLIEGENTHART, J.F.G. (1992) *Carbohydrate Research*, **231**, 273.

GRUTER, M., LEEFLANG, B.R., KUIPER, J., KAMERLING, J.P. and VLIEGENTHART, J.F.G. (1993) *Carbohydrate Research*, **239**, 209.
GÜNDÜZ, U. and REJAEE, R. (1997) *Turkish Journal of Biology*, **21**, 109.
GUPTA, P.K., MITAL, K.B., GARG, K.S. and MISHRA, P.O. (1994) *Journal of Food Biochemistry*, **18**, 55.
GYOSHEVA, B. (1985) *Nahrung*, **29**, 185.
GYOSHEVA, B. (1986) *Dairy Science Abstracts*, **48**, 564.
GYOSHEVA, B., PETROVA, I. and MUTAFCHIEVA, M. (1996) *Nahrung*, **40**, 68.
HABIBI-NAJAFI, M.B. and LEE, B.H. (1994a) *Applied Microbiology and Biotechnology*, **42**, 280.
HABIBI-NAJAFI, M.B. and LEE, B.H. (1994b) *Journal of Dairy Science*, **77**, 385.
HABIBI-NAJAFI, M.B. and LEE, B.H. (1995) *Journal of Dairy Science*, **78**, 251.
HAESOO, K., JOUNGJWA, A., KYUNGWOOK, L. and DONGSOON, S. (1996) *Foods and Biotechnology*, **5**, 94.
HAGGERTY, R.J., LUEDECKE, L.O., NAGEL, C.W. and MASSEY, L.K. (1984) *Journal of Food Science*, **49**, 1194.
HEBERT, E.M., FERNÁNDEZ MURGA, M.L., FONT DE VALDEZ, G. and DE GIORI, G.S. (1998) *Milchwissenschaft*, **53**, 184.
HEGAZI, F.Z. (1987) *Nahrung*, **31**, 19.
HEGAZI, F.Z. and ABO-ELNAGA, I.G. (1989) *Deutsche Lebensmittel-Rundschau*, **85**, 322.
HEGAZI, F.Z. and ABO-ELNAGA, I.G. (1990) *Microbiologie, Aliments, Nutrition*, **8**, 119.
HEMME, D. and NARDI, M. (1980) *Lait*, **60**, 375.
HEMME, D., VASSAL, L., FOYEN, H. and AUCLAIR, J.P. (1979) *Lait*, **59**, 597.
HEMME, D., WAHL, D. and NARDI, M. (1980) *Lait*, **60**, 111.
HEMME, D., NARDI, M. and WAHL, D. (1981) *Lait*, **61**, 1.
HICKEY, M.W., HILLIER, A.J. and JAGO, G.R. (1983a) *Australian Journal of Dairy Technology*, **38**, 118.
HICKEY, M.W., HILLIER, A.J. and JAGO, G.R. (1983b) *Australian Journal of Dairy Technology*, **38**, 154.
HICKEY, M.W., HILLIER, A.J. and JAGO, G.R. (1986) *Applied and Environmental Microbiology*, **51**, 825.
HOLCK, A. and NAES, H. (1991) *Journal of General Microbiology*, **138**, 1353.
HOLLAND, B., UNWIN, I.D. and BUSS, D.H. (1989) In *Milk Products and Eggs, 4th Supplement to McCance & Widdowson's The Composition of Foods*, 4th Edition, Royal Society of Chemistry, Cambridge.
HOPPNER, K. and LAMPI, B. (1990) *Canadian Institute of Food Science and Technology Journal*, **23**, 223.
HOSONO, A., ZHANG, X. and OTANI, H. (1989) *Japanese Journal of Dairy and Food Science*, **38**, A9.
HOSONO, A., SUZUKI, M. and OTANI, H. (1991) *Animal Science and Technology*, **62**, 39.
HRUSKAR, M., VAHCIC, N. and RITZ, M. (1995) *Mljekarstvo*, **45**, 175.
HUTKINS, R.W. and MORRIS, H.A. (1987) *Journal of Food Protection*, **50**, 876.
HUTKINS, R.W. and NANNEN, N.L. (1993) *Journal of Dairy Science*, **76**, 2354.
HUTKINS, R.W. and PONNE, C. (1991) *Applied and Environmental Microbiology*, **57**, 941.
HUTKINS, R.W., MORRIS, H.A. and MCKAY, L.L. (1985a) *Applied and Environmental Microbiology*, **50**, 772.
HUTKINS, R.W., MORRIS, H.A. and MCKAY, L.L. (1985b) *Applied and Environmental Microbiology*, **50**, 777.
ILINOVA, P. and NAUMOVA, R. (1984) *Dairy Science Abstracts*, **46**, 963.
IMHOF, R., GLÄTTLI, H. and BOSSET, J.O. (1994) *Lebensmittel-Wissenschaft und Technologie*, **27**, 442.
IMHOF, R., GLÄTTLI, H. and BOSSET, J.O. (1995) *Lebensmittel-Wissenschaft und Technologie*, **28**, 78.
ISMAIL, A.A., SALAMA, F.A., ABOU-DONIA, S.A., KHADER, A.E. and ABOU-ZEID, N.A. (1980) *Mounoufeia Journal of Agricultural Research*, **3**, 211.
KAHALA, M., PAHKALA, E. and PIHLANTO-LEPÄLÄ, A. (1993) *Agricultural Science of Finland*, **2**, 379.
KALANTZOPOULOS, G., TSAKALIDOU, E. and GEORGALA, C. (1990a) In *Processing and Quality of Foods*, Vol. 2, Ed. by Zeutheu, P., Cheftel, J.C., Eriksson, C., Gormley, T.R., Links, P. and Paulus, K. Elsevier Applied Science, London, pp. 2.52–2.60.
KALANTZOPOULOS, G., TSAKALIDOU, E. and MANOLOPOULOU, E. (1990b) *Journal of Dairy Research*, **57**, 593.
KANATANI, K. and OSHIMURA, K. (1994) *Journal of Fermentation and Bioengineering*, **78**, 123.
KANDLER, O. (1983) *Antonie van Leeuwenhoek*, **49**, 209.
KANEKO, T., SUZUKI, H. and TAKAHASHI, T. (1987) *Dairy Science Abstracts*, **49**, 201.
KANG, Y., FRANK, J.F. and LILLARD, D.A. (1988) *Cultured Dairy Products Journal*, **23**(4), 6.
KEENAN, T.W. and BILLS, D.D. (1968) *Journal of Dairy Science*, **51**, 1561.
KHALID, N.M. and MARTH, E.H. (1990a) *Applied and Environmental Microbiology*, **56**, 381.
KHALID, N.M. and MARTH, E.H. (1990b) *Systematic and Applied Microbiology*, **13**, 311.
KHALID, N.M., EL-SODA, M. and MARTH, E.H. (1990) *Journal of Dairy Science*, **73**, 2711.
KHALID, N.M., EL-SODA, M. and MARTH, E.H. (1991) *Journal of Dairy Science*, **74**, 29.
KHAMAGACHEVA, N.S., KHAMANACHEVA, N.I. and SHAROBAIKO, V.I. (1988) *Dairy Science Abstracts*, **50**, 241.
KIELWEIN, G. and DAUN, U. (1980) *Dairy Science Abstracts*, **42**, 361.
KILARA, A. and SHAHANI, K.M. (1976) *Journal of Dairy Science*, **59**, 2031.
KILARA, A. and SHAHANI, K.M. (1978) *Journal of Dairy Science*, **61**, 1793.
KIM, S.I., NALASKOWSKA, M., GERMOND, J.E., PRIDMORE, D. and SÖLL, D. (1996) *Nucleic Acid Research*, **24**, 2648.
KIMMEL, S.A., ROBERTS, R.F. and ZIEGLER, G.R. (1998) *Applied and Environmental Microbiology*, **64**, 659.
KLAENHAMMER, T.R. (1995) *International Dairy Journal*, **5**, 1019.
KLUPSCH, H.J. (1984) German Federal Republic Patent Application, DE 33 00 123 A1.
KNEIFEL, W., HOLUB, S. and WIRTHMANN, M. (1989) *Journal of Dairy Research*, **56**, 651.
KNEIFEL, W., ERHARD, F. and JAROS, D. (1991) *Milchwissenschaft*, **46**, 685.

KNEIFEL, W., ULBERTH, F., ERHARD, F. and JAROS, D. (1992) *Milchwissenschaft*, **47**, 362.
KOCHHAR, S., CHUARD, N. and HOTTINGER, H. (1992a) *Biochemical and Biophysical Research Communications*, **185**, 705.
KOCHHAR, S., CHUARD, N. and HOTTINGER, H. (1992b) *Journal of Biological Chemistry*, **267**, 20298.
KOCHHAR, S., HUNZINKER, P.E., LEONG-MORGENTHALER, P. and HOTTINGER, H. (1992c) *Biochemical and Biophysical Research Communications*, **184**, 60.
KOCHHAR, S., HUNZINKER, P.E., LEONG-MORGENTHALER, P. and HOTTINGER, H. (1992d) *Journal of Biological Chemistry*, **267**, 8499.
KOJIC, M., FIRA, D., BANINA, A. and TOPISIROVIC, L. (1991) *Applied and Environmental Microbiology*, **57**, 1753.
KOK, J. (1990) *FEMS Microbiology Reviews*, **87**, 15.
KONDRATENKO, M. and GYOSHEVA, B. (1985) *Dairy Science Abstracts*, **47**, 744.
KOTZ, C.M., FURNE, J.K., SAVAIANO, D.A. and LEVITT, M.D. (1994) *Journal of Dairy Science*, **77**, 3538.
KRSEV, L. (1976) *Dairy Science Abstracts*, **38**, 688.
KUNATH, P. and VON KANDLER, O. (1980) *Milchwissenschaft*, **35**, 470.
KUNJI, E.R.S., MIERAU, I., HAGTING, A., POOLMAN, B. and KONINGS, W.N. (1996) *Antonie van Leeuwenhoek*, **70**, 187.
KYRIAKIDIS, S.M., SAKELLARIS, G. and SOTTROUDIS, T.G. (1993) *Letters in Applied Microbiology*, **16**, 295.
LALOI, P., ATLAN, D., BLANC, B., GILBERT, C. and PORTALIER, R. (1991) *Applied Microbiology and Biotechnology*, **36**, 196.
LARSON, B.L. and HEGARTY, H.M. (1979) *Journal of Dairy Science*, **62**, 1641.
LAUKKANEN, M., ANTILA, P., ANTILA, V. and SALMINEN, K. (1988) *Meijertieteellinen Aikakauskirja*, **XLVI**, 7.
LAVANCHY, P. and STEIGER, G. (1984) *Special Publication – Royal Society of Chemistry*, No. 49, pp. 317–318.
LAW, J. and HAANDRIKMAN, A. (1997) *International Dairy Journal*, **7**, 1.
LAWRENCE, R.C., THOMAS, T.D. and TERZAGHI, B.E. (1976) *Journal of Dairy Research*, **43**, 141.
LAYE, I., KARLESKIND, D. and MORR, C.V. (1993) *Journal of Food Science*, **58**, 991.
LEE, K.D., LO, C.R., RICHTER, R.L. and DILL, C.W. (1995) *Journal of Dairy Science*, **78**, 2666.
LEES, G.J. and JAGO, G.R. (1966) In *Proceedings of Australian Dairy Manufacture Research Seminar*, April 1966, Melbourne, Australia.
LEES, G.J. and JAGO, G.R. (1976a) *Journal of Dairy Research*, **43**, 63.
LEES, G.J. and JAGO, G.R. (1976b) *Journal of Dairy Research*, **43**, 75.
LEES, G.J. and JAGO, G.R. (1977) *Journal of Dairy Research*, **44**, 139.
LEES, G.J. and JAGO, G.R. (1978a) *Journal of Dairy Science*, **61**, 1205.
LEES, G.J. and JAGO, G.R. (1978b) *Journal of Dairy Science*, **61**, 1216.
LEMOINE, J., CHIRAT, F., WIERUSZESKI, J.M., STRECKER, G., FAVRE, N. and NEESER, J.R. (1997) *Applied and Environmental Microbiology*, **63**, 3512.
LENTNER, C. (Ed.) (1984) In *Geigy Scientific Tables – Physical Chemistry, Composition of Blood, Hematology, Somatometric Data*, Vol. 3, 8th Edition, Ciba-Geigy, Basle.
LENTNER, C. (Ed.) (1986) In *Geigy Scientific Tables – Biochemistry, Metabolism of Xenobiotics, Inborn Errors of Metabolism, Pharmacogenetics and Ecogenetics*, Vol. 4, 8th Edition, Ciba-Geigy, Basle.
LINK, H. and PAHUD, J.J. (1991) European Patent Application, EP 0 432 490 A2.
LINKO, S., ENWALD, S., ZHU, Y.H. and MÄYRÄ-MÄKINEN, A. (1998) *Journal of Industrial Microbiology and Biotechnology*, **20**, 215.
LIRA, F., GARCIA-GARIBAY, M., WACHER-RODARTE, C., FARRES, A. and MARSHALL, V.M.E. (1997) *International Dairy Journal*, **7**, 279.
LOPEZ-FANDINO, R. and ARDÖ, Y. (1991) *Journal of Dairy Reserach*, **58**, 469.
LUCA, C. (1974) *Dairy Science Abstracts*, **36**, 633.
LUCZYNSKA, A., BIJOK, F., WAJNERT, T., KAZIMIERCZAK, W., LIPINSKA, E., KOSIKOWSKA, M. and JAKUBCZYK, E. (1978) *XX International Dairy Congress*, **E**, 836.
MACHUGA, E.J. and IVES, D.H. (1984) *Biochimica et Biophysica Acta*, **789**, 26.
MALAN, A.L. (1987) *Dairy Science Abstracts*, **49**, 610.
MALIK, R.K., PRASHER, R. and MATHER, D.K. (1994) *Indian Journal of Dairy Science*, **47**, 987.
MANCA DE NADRA, M.C., PESCE DE RUIZ HOLGADO, A.A. and OLIVER, G. (1982) *Milchwissenschaft*, **37**, 669.
MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A. and OLIVER, G. (1985) *Milchwissenschaft*, **40**, 409.
MANCA DE NADRA, M.C., RAYA, R.R., PESCE DE RUIZ HOLGADO, A.A. and OLIVER, G. (1987) *Milchwissenschaft*, **42**, 92.
MANCA DE NADRA, M.C., AMOROSO, M.J. and OLIVER, G. (1988) *Microbiologie, Aliments, Nutrition*, **6**, 269.
MANJUNATH, N., JOSEPH, A.M. and SRINIVASAN, R.A. (1983) *Egyptian Journal of Dairy Science*, **11**, 111.
MARRANZINI, R.M., SCHMIDT, R.H., SHIREMAN, R.B., MARSHALL, M.R. and CORNELL, J.A. (1989) *Journal of Dairy Science*, **72**, 1142.
MARSHALL, V.M.E. (1987) *FEMS Microbiology Reviews*, **46**, 327.
MARSHALL, V.M.E. and COLE, V.M. (1983) *Journal of Dairy Research*, **50**, 375.
MARSHALL, V.M.E. and TAMIME, A.Y. (1997a) In *Microbiology and Biochemistry of Cheese and Fermented Milk*, 2nd Edition, Ed. by Law, B.A., Blackie Academic & Professional, London, pp. 153–192.
MARSHALL, V.M.E. and TAMIME, A.Y. (1997b) *International Journal of Dairy Technology*, **50**, 35.

MARTIN-HERNÁNDEZ, M.C., ALTING, A.C. and EXTERKATE, F.A. (1994) *Applied Microbiology and Biotechnology*, **40**, 828.
MCKAY, L.L., WALTER, L.A., SANDINE, W.E. and ELLIKER, P.R. (1969) *Journal of Bacteriology*, **99**, 603.
MEISEL, H., GOEPFERT, A. and GÜNTHER, S. (1997) *Milchwissenschaft*, **52**, 307.
MEYER, J. and JORDI, R. (1987) *Journal of Dairy Science*, **70**, 738.
MIDWINTER, R.G. and PRITCHARD, G.G. (1994) *Journal of Applied Bacteriology*, **77**, 288.
MILLER, I. and KANDLER, O. (1967a) *Milchwissenschaft*, **22**, 150.
MILLER, I. and KANDLER, O. (1967b) *Milchwissenschaft*, **22**, 608.
MITIC, S., OTENHAJMER, I. and OBRADOVIC, D. (1974) *Dairy Science Abstracts*, **36**, 656.
MIYAKAWA, H., KOBAYASHI, S., SHIMAMURA, S. and TOMITA, M. (1991) *Journal of Dairy Science*, **74**, 2375.
MIYAKAWA, H., KOBAYASHI, S., SHIMAMURA, S. and TOMITA, M. (1992) *Journal of Dairy Science*, **75**, 27.
MOGENSEN, G. (1992) In *Food Technology Europe*, Ed. by Turner, A., Sterling Publications, London, pp. 35–39.
MOLLET, B. (1996) *CEREVISA – Belgian Journal of Brewing and Biotechnology*, **21**, 63.
MONNET, V., CONDON, S., COGAN, T.M. and GRIPON, J.C. (1996) In *Dairy Starter Cultures*, Ed. by Cogan, T.M. and Accolas, J.-P., VCH Publishers, New York, pp. 47–99.
MOON, Y.I. and KIM, Y.K. (1986) *Dairy Science Abstracts*, **48**, 242.
MOON, Y.I. and KIM, Y.K. (1990a) *Dairy Science Abstracts*, **52**, 203.
MOON, Y.I. and KIM, Y.K. (1990b) *Dairy Science Abstracts*, **52**, 203.
MOON, Y.I., KIM, M.B. and KIM, Y.K. (1989a) *Dairy Science Abstracts*, **51**, 135.
MOON, Y.I., KIM, M.B. and KIM, Y.K. (1989b) *Dairy Science Abstracts*, **51**, 135.
MORICHI, T., SHARPE, M.E. and REITER, B. (1968) *Journal of General Microbiology*, **53**, 405.
MOZZI, F., SAVOY DE GIORI, G., OLIVER, G. and FONT DE VALDEZ, G. (1994) *Milchwissenschaft*, **49**, 667.
MOZZI, F., OLIVER, G., SAVOY DE GIORI, G. and FONT DE VALDEZ, G. (1995a) *Milchwissenschaft*, **50**, 80.
MOZZI, F., SAVOY DE GIORI, G., OLIVER, G. and FONT DE VALDEZ, G. (1995b) *Milchwissenschaft*, **50**, 186.
MOZZI, F., SAVOY DE GIORI, G., OLIVER, G. and FONT DE VALDEZ, G. (1995c) *Milchwissenschaft*, **50**, 307.
MOZZI, F., SAVOY DE GIORI, G., OLIVER, G. and FONT DE VALDEZ, G. (1996) *Milchwissenschaft*, **51**, 670.
MOZZI, F., SAVOY DE GIORI, G., OLIVER, G. and FONT DE VALDEZ, G. (1997) *Milchwissenschaft*, **52**, 663.
MULDER, H. and WALSTRA, P. (1974) In *The Milk Fat Globule Emulsion Science as Applied to Milk Products and Comparable Foods*, Commonwealth Agricultural Bureaux, Farnham Royal.
MULHOLLAND, F. (1994) In *Biochemistry of Milk Products*, Ed. by Andrews, A.T. and Varley, J., Royal Society of Chemistry, Cambridge, pp. 83–93.
MUTAI, M., ASO, K. and SHIROTA, M. (1972) In *Conversion and Manufacture of Foodstuffs by Microorganisms*, Yakult Institute, Saikon Publishing, Japan.
NACHEV, L. (1970) *Dairy Science Abstracts*, **32**, 181.
NADATHUR, S.R., CARNEY, J.R., GOULD, S.J. and BAKALINSKY, A.T. (1996) *Mutation Research, Environmental Mutagenesis and Related Subjects*, **359**, 179.
NADER DE MACIAS, M.E., PERDIGON, G., OLIVER, G. and PESCE DE RUIZ HOLGADO, A.A. (1986) *Systematic and Applied Microbiology*, **8**, 28.
NAES, H. and NISSEN-MEYER, J. (1992) *Journal of General Microbiology*, **138**, 313.
NAKAI, T. and ELLIOT, J.A. (1965) *Journal of Dairy Science*, **48**, 287.
NAKAJIMA, H., TOYODA, S., TOBA, T., ITOH, T., MUKAI, T., KITAZAWA, H. and ADACHI, S. (1990) *Journal of Dairy Science*, **73**, 1472.
NANNEN, N.L. and HUTKINS, R.W. (1991a) *Journal of Dairy Science*, **74**, 741.
NANNEN, N.L. and HUTKINS, R.W. (1991b) *Journal of Dairy Science*, **74**, 747.
NAVDER, K.P., HUANG, R.S., FRYER, E.B. and FRYER, H.C. (1990) *Journal of Food Science*, **55**, 585.
NODA, H., AKASAKA, N. and OHSUGI, M. (1994) *Journal of Nutritional Science and Vitaminology*, **40**, 181.
NOH, D.O. and GILLILAND, S.E. (1994) *Journal of Dairy Science*, **77**, 3532.
OBERG, C.J., WEIMER, B.C., MOYES, L.V., BROWN, R.J. and RICHARDSON, G.H. (1991) *Journal of Dairy Science*, **74**, 398.
OCCHINO, L.A., MORRIS, H.A. and SAVIANO, D.A. (1986) *Journal of Dairy Science*, **69**, 2583.
OHMIYA, K. and SATO, Y. (1968) *Journal of Agricultural and Biological Chemistry*, **32**, 291.
OHMIYA, K. and SATO, Y. (1969) *Journal of Agricultural and Biological Chemistry*, **33**, 669.
OHMIYA, K. and SATO, Y. (1978) *Journal of Agricultural and Biological Chemistry*, **42**, 7.
OKONKWO, P. and KINSELLA, J.E. (1969a) *Journal of Dairy Science*, **52**, 1861.
OKONKWO, P. and KINSELLA, J.E. (1969b) *American Journal of Clinical Nutrition*, **22**, 532.
ONER, M.D. and ERICKSON, L.E. (1986) *Biotechnology and Bioengineering*, **28**, 883.
ONER, M.D., ERICKSON, L.E. and YANG, S.S. (1986a) *Biotechnology and Bioengineering*, **28**, 895.
ONER, M.D., ERICKSON, L.E. and YANG, S.S. (1986b) *Biotechnology and Bioengineering*, **28**, 902.
ONER, M.D., ERICKSON, L.E. and YANG, S.S. (1986c) *Biotechnology and Bioengineering*, **28**, 919.
ONER, M.D., ERICKSON, L.E. and YANG, S.S. (1986d) In *Food Engineering and Process Applications*, Vol. 1, Ed. by Le Maguer, M. and Jelen, P., Elsevier Applied Science, London, pp. 367–376.
ORDONEZ, G.O. and JEON, I.T. (1995) *Cultured Dairy Products Journal*, **30**(4), 29.
OTT, A., FAY, L.B. and CHAINTREAU, A. (1997) *Journal of Agricultural and Food Chemistry*, **45**, 850.
OTTERHOLM, A., ORDAL, Z.J. and WITTER, L.D. (1968) *Applied Microbiology*, **16**, 524.

OTTOGALLI, G., RISMINI, R., RONDIMINI, G. and SARACCHI, S. (1974) *Dairy Science Abstracts*, **36**, 314.
PERMI, L., SANDINE, W.E. and ELLIKER, P.R. (1972) *Applied Microbiology*, **24**, 51.
PETIT, C., GRILL, J.P., MAAZOUZI, N. and MARCZAK, R. (1991) *Applied Microbiology and Biotechnology*, **36,** 216.
PETTE, J.W. and LOLKEMA, J. (1950) *Netherlands Milk and Dairy Journal*, **4**, 261.
POCH, M.T. and SOMKUTI, G.A. (1998) *Biotechnology Techniques*, **7**, 781.
POOLMAN, B. (1993) *Lait*, **73**, 87.
POOLMAN, B., ROYER, T.J., MAINZER, S.E. and SCHMIDT, B.F. (1989) *Journal of Bacteriology*, **171**, 244.
POOLMAN, B., ROYER, T.J., MAINZER, S.E. and SCHMIDT, B.F. (1990) *Journal of Bacteriology*, **172**, 4037.
POOLMAN, B., KNOL, J. and LOLKEMA, J.S. (1995) *Journal of Biological Chemistry*, **270**, 12995.
POZNANSKI, S., LENOIR, J. and MOCQUOT, G. (1965) *Lait*, **43**, 3.
PRAKASH, B.S. and SHARMA, R.S. (1986) *Journal of Food Science and Technology – India*, **23**, 85.
PRITCHARD, G.G. and COOLBEAR, T. (1993) *FEMS Microbiology Reviews*, **12**, 179.
PUHAN, Z., BANHEGYI, M. and FLÜELER, O. (1973a) *Schweizerische Milchwirtschaftliche Forschung*, **2**, 53.
PUHAN, Z., FLÜELER, O. and BANHEGYI, M. (1973b) *Schweizerische Milchwirtschaftliche Forschung*, **2**, 53.
PUHAN, Z., FLÜELER, O. and BANHEGYI, M. (1974) *XIX International Dairy Congress*, **IE**, 451.
PULUSANI, S.R., RAO, D.R. and SUNKI, G.R. (1979) *Journal of Food Science*, **44**, 575.
RABIER, D. and DESMAZEAUD, M.J. (1973) *Biochimie*, **55**, 389.
RAMOS, M.S. and HARLANDER, S.K. (1990) *Applied Microbiology and Biotechnology*, **34**, 368.
RANTANEN, T. and PALVA, A. (1997) *Microbiology – Reading*, **143**, 3899.
RAO, M.V.R. and DUTTA, S.M. (1977) *Journal of Applied and Environmental Microbiology*, **34**, 185.
RAO, M.V.R. and DUTTA, S.M. (1978) *XX International Dairy Congress*, **E**, 495.
RAO, D.R. and PULUSANI, S.R. (1981) *Journal of Food Science*, **46**, 630.
RAO, D.R. and REDDY, J.C. (1984) *Journal of Food Science*, **49**, 748.
RAO, D.R. and SHAHANI, K.M. (1987) *Cultured Dairy Products Journal*, **22**(1), 6.
RAO, D.R., REDDY, A.V., PULUSANI, S.R. and CORNWELL, P.E. (1984) *Journal of Dairy Science*, **67**, 1169.
RASIC, J. and PANIC, B. (1963) *Dairy Industries*, **28**, 35.
RASIC, J. and VUCUROVIC, N. (1973) *Milchwissenschaft*, **28**, 220.
RASIC, J., STOJSLAVLJEVIC, T. and CURCIC, R. (1971a) *Milchwissenschaft*, **26**, 219.
RASIC, J., CURCIC, R., STOJSLAVLJEVIC, T. and OBRADOVIC, B. (1971b) *Milchwissenschaft*, **26**, 496.
RASIC, J., VUCUROVIC, N. and OBRADOVIC, B. (1973) *Milchwissenschaft*, **28**, 168.
RAWSON, H.L. and MARSHALL, V.M.E. (1997) *International Journal of Food Science and Technology*, **32**, 213.
RAYA, R.R., MANCA DE NADRA, M.C., PESCE DE RUIZ HOLGADO, A. and OLIVER, G. (1986a) *Milchwissenschaft*, **41**, 397.
RAYA, R.R., MANCA DE NADRA, M.C., PESCE DE RUIZ HOLGADO, A. and OLIVER, G. (1986b) *Milchwissenschaft*, **41**, 630.
REDDY, G.V., SHAHANI, K.M. and BANERGEE, M.R. (1973a) *Journal of the National Cancer Institute*, **50**, 815.
REDDY, M.S., WILLIAMS, F.D. and REINBOLD, G.W. (1973b) *Journal of Dairy Science*, **56**, 634.
REDDY, K.P., SHAHANI, K.M. and KULKARNI, S.M. (1976) *Journal of Dairy Science*, **59**, 191.
REPS, A., HAMMOND, E.G. and GLATZ, B.A. (1987) *Journal of Dairy Science*, **70**, 559.
REZANKA, T., VIDEN, I., POKORNA, L., KLANOVA, K. and PODOJIL, M. (1983) *Folia Microbiologica*, **28**, 470.
RICHMOND, M.L., HARTE, B.R., GRAY, J.I. and STINE, C.M. (1987) *Journal of Dairy Science*, **70**, 1140.
ROBERTS, C.M., FETT, W.F., OSMAN, S.F., WIJEY, C., O'CONNOR, J.V. and HOOVER, D.G. (1995) *Journal of Applied Bacteriology*, **78**, 463.
ROBIJN, G.W., THOMAS, J.R., HAAS, H., VAN DEN BERG, D.J.C., KAMERLING, J.P. and VLIEGENTHART, J.F.G. (1995a) *Carbohydrate Research*, **276**, 137.
ROBIJN, G.W., VAN DEN BERG, D.J.C., HAAS, H., KAMERLING, J.P. and VLIEGENTHART, J.F.G. (1995b) *Carbohydrate Research*, **276**, 117.
ROBIJN, G.W., WIENK, H.L.T., VAN DEN BERG, D.J.C., HAAS, H., KAMERLING, J.P. and VLIEGENTHART, J.F.G. (1996) *Carbohydrate Research*, **285**, 129.
ROBINSON, R.K., TAMIME, A.Y. and CHUBB, L.W. (1977) *The Milk Industry*, **79**(4), 4.
RODWELL, V. (1975) In *Review of Physiological Chemistry*, 15th Edition, Ed. by Harper, H.A., Lange Medical Publications, California.
RUL, F. and MONNET, V. (1997) *Journal of Applied Microbiology*, **82**, 695.
RUL, F., MONNET, V. and GRIPON, J.-C. (1994) *Journal of Dairy Science*, **77**, 2880.
RYSSTAD, G. and ABRAHAMSEN, R.K. (1987) *Journal of Dairy Research*, **54**, 257.
SAIDI, B. and WARTHESEN, J.J. (1989) *Journal of Dairy Science*, **72**, 2900.
SAIDI, B. and WARTHESEN, J.J. (1993) *International Dairy Journal*, **3**, 675.
SANDINE, W.E. and ELLIKER, P.R. (1970) *Journal of Agriculture and Food Chemistry*, **18**, 557.
SATO, Y. and NAKASHIMA, J. (1965) *Journal of Agricultural Chemical Society – Japan*, **39**, 292.
SCHELLHAAS, S.M. (1984) *Dissertation Abstracts International B*, **44**, 2698.
SCHELLHAAS, S.M. and MORRIS, H.A. (1985) *Food Microstructure*, **4**, 279.
SCHMIDT, R.H., DAVIDSON, S.M. and BATES, R.P. (1983) *Journal of Food Science*, **48**, 1556.
SCHMIDT, R.H., KENNEDY, L.B., MCMULLAN, E.B. and MASON, E.R. (1989) *Journal of Agricultural and Food Chemistry*, **37**, 1215.

SCHULZ, M.E. and HINGST, G. (1954) *Milchwissenschaft*, **9**, 330.
SCOTT, K.J. and BISHOP, D.R. (1986) *Journal of the Society of Dairy Technology*, **39**, 32.
SEBASTIANI, H. and ZELGER, G. (1998) *Milchwissenschaft*, **53**, 15.
SENECA, H., HENDERSON, E. and COLLINS, A. (1950) *American Practitioner for Digestive Treatment*, **1**, 1252.
SHAH, N. and JELEN, P. (1991) *Milchwissenschaft*, **46**, 570.
SHAHANI, K.M., REDDY, G.V. and JOE, A.M. (1974) *XIX International Dairy Congress*, **IE**, 569.
SHAHBAL, S., HEMME, D. and DESMAZEAUD, M. (1991) *Lait*, **71**, 351.
SHANKAR, P.A. (1977) In *Inter-relationship of* S. thermophilus *and* L. bulgaricus *in Yoghurt Cultures*, PhD Thesis, University of Reading, Reading.
SHANKAR, P.A. and DAVIES, F.L. (1978) *XX International Dairy Congress*, **E**, 467.
SHARMA, R.K. and DUTTA, S.M. (1990) *Indian Journal of Dairy Science*, **43**, 213.
SHARMA, R., LAL, D. and MALIK, R.K. (1996) *Indian Journal of Dairy and Biosciences*, **7**, 1.
SHARPE, M.E., GARVIE, E.I. and TILBURY, R.H. (1972) *Applied Microbiology*, **23**, 389.
SHIDLOVSKAYA, V.V. and DYACHENKO, P.F. (1968) *Dairy Science Abstracts*, **30**, 271.
SIKKEMA, J. and OBA, T. (1998) *Snow Brand R&D Reports*, No. **107**, pp. 1–31.
SINGH, J. (1983) *Milchwissenschaft*, **38**, 347.
SINGH, J. and KAUL, A. (1982a) *Egyptian Journal of Dairy Science*, **10**, 129.
SINGH, J. and KAUL, A. (1982b) *Folia Microbiologica*, **27**, 142.
SINGH, J. and RANGANATHAN, B. (1974a) *XIX International Dairy Congress*, **IE**, 385.
SINGH, J. and RANGANATHAN, B. (1974b) *Acta Microbiologica Polonica*, **6**, 15.
SINGH, J. and RANGANATHAN, B. (1977a) *Indian Journal of Dairy Science*, **30**, 70.
SINGH, J. and RANGANATHAN, B. (1977b) *Indian Journal of Experimental Biology*, **15**, 490.
SINGH, J. and RANGANATHAN, B. (1978) *Journal of Dairy Research*, **45**, 123.
SINGH, J. and RANGANATHAN, B. (1979) *Milchwissenschaft*, **34**, 288.
SINGH, J. and SHARMA, D.K. (1983) *Milchwissenschaft*, **38**, 148.
SINGH, J., RANGANATHAN, B. and CHANDER, H. (1978) *XX International Dairy Congress*, **E**, 517.
SINGH, J., KHANNA, A. and CHANDER, H. (1980) *Journal of Food Protection*, **43**, 399.
SINGH, J., SHARMA, D.K. and JAIN, L.K. (1982) *Egyptian Journal of Dairy Science*, **10**, 125.
SINHA, R.P., MODLER, H.W. and EMMONS, D.B. (1989) *Cultured Dairy Products Journal*, **24**(2), 12.
SKRIVER, A., BUCHHEIM, W. and QVIST, K.B. (1995) *Milchwissenschaft*, **50**, 683.
SMART, J. (1991) *Applied Microbiology and Biotechnology*, **34**, 495.
SMART, J. and RICHARDSON, B. (1987) *Applied Microbiology and Biochemistry*, **26**, 177.
SMART, J.B., CROW, V.L. and THOMAS, T.D. (1985) *New Zealand Journal of Dairy Science and Technology*, **20**, 43.
SOMKUTI, G.A. and STEINBERG, D.H. (1978) *Journal of Dairy Science*, **61** (Suppl. 1), 118.
SOMKUTI, G.A. and STEINBERG, D.H. (1979a) *Journal of Applied Biochemistry*, **1**, 357.
SOMKUTI, G.A. and STEINBERG, D.H. (1979b) *Journal of Food Protection*, **42**, 881.
SOMKUTI, G.A. and STEINBERG, D.H. (1991) *Biotechnology Letters*, **13**, 809.
SOMKUTI, G.A. and STEINBERG, D.H. (1995) *Biotechnology and Applied Biochemistry*, **21**, 23.
STANIER, R.Y., INGRAM, J.L., WHEELIS, M.L. and PAINTER, P.R. (1987) In *The Microbial World*, 5th Edition, MacMillan Education, London.
STEFANITSI, D. and GAREL, J.-R. (1997) *Letters in Applied Microbiology*, **24**, 180.
STEFANITSI, D., SAKELLARIS, G. and GAREL, J.-R. (1995) *FEMS Microbiology Letters*, **128**, 53.
STEFANOVA, M. and GYOSHEVA, B. (1985) *Dairy Science Abstracts*, **47**, 899.
STINGELE, F., NEESER, J.-R. and MOLLET, B. (1996) *Journal of Bacteriology*, **178**, 1680.
STOJSLAVLJEVIC, T., RASIC, J. and CURCIC, R. (1971) *Milchwissenschaft*, **26**, 147.
SUZUKI, Y. (1990) *Dairy Science Abstracts*, **52**, 380.
SUZUKI, I., WATANABA, M., KITADA, T., KATO, S. and MORICHI, T. (1979) *Japanese Journal of Zootechnical Science*, **50**, 796.
SUZUKI, I., KATO, S., KITADA, T., YANO, N. and MORICHI, T. (1986) *Journal of Dairy Science*, **69**, 971.
TAGUCHI, H. and OHTA, T. (1991) *Journal of Biological Chemistry*, **266**, 12588.
TAGUCHI, H. and OHTA, T. (1993) *Journal of Biological Chemistry*, **268**, 18030.
TAKAHASHI, T. and MOROTOMI, M. (1994) *Journal of Dairy Science*, **77**, 3275.
TAMIME, A.Y. (1977a) In *Some Aspects of the Production of Yoghurt and Condensed Yoghurt*, PhD Thesis, University of Reading, Reading.
TAMIME, A.Y. (1977b) *Dairy Industries International*, **42**(8), 7.
TAMIME, A.Y. (1978) *Cultured Dairy Products Journal*, **13**(3), 16.
TAMIME, A.Y. and DEETH, H.C. (1980) *Journal of Food Protection*, **43**, 939.
TAMIME, A.Y. and ROBINSON, R.K. (1978) *Milchwissenschaft*, **33**, 209.
TAMIME, A.Y., KALAB, M. and DAVIES, G. (1984) *Food Microstructure*, **3**, 83.
TAN, P.S.T., SASAKI, M., BOSMAN, B.W. and IWASAKI, T. (1995) *Applied and Environmental Microbiology*, **61**, 3430.
TANEV, G. and ZIVKOVA, A. (1977) *Milchwissenschaft*, **32**, 280.
TEGGATZ, J.A. and MORRIS, H.A. (1990) *Food Structure*, **9**, 133.
THOMAS, T.D. and CROW, V.L. (1984) *Applied and Environmental Microbiology*, **48**, 186.

THOMAS, T.D. and PRITCHARD, G.G. (1987) *FEMS Microbiology Reviews*, **46**, 245.
THOMOPOULOS, C., TZIA, C. and MILKAS, D. (1993) *Milchwissenschaft*, **48**, 426.
THOMPSON, J. (1988) *Biochimie*, **70**, 325.
TINSON, W., HILLIER, A.J. and JAGO, G.R. (1982a) *Australian Journal of Dairy Technology*, **37**, 8.
TINSON, W., HILLIER, A.J. and JAGO, G.R. (1982b) *Australian Journal of Dairy Technology*, **37**, 22.
TINSON, W., BROOME, M.C., HILLIER, A.J. and JAGO, G.R. (1982c) *Australian Journal of Dairy Technology*, **37**, 14.
TOBA, T., TOMRRA, Y., ITOH, T. and ADACHI, S. (1981) *Journal of Dairy Science*, **64**, 185.
TOMÉ, D. (1998) In *Danone World Newsletter*, No. **17**, pp. 3–13.
TRUFFA-BACHI, P. and COHEN, G.N. (1968) *Annual Review of Biochemistry*, **37**, 79.
TSAKALIDOU, E. and KALANTZOPOULOS, G. (1992) *Journal of Applied Bacteriology*, **72**, 227.
TSAKALIDOU, E., ZOIDOU, E. and KALANTZOPOULOS, G. (1992) *Milchwissenschaft*, **47**, 296.
TSAKALIDOU, E., DALEZIOS, I., GEORGALAKI, M. and KALANTZOPOULOS, G. (1993) *Journal of Dairy Science*, **76**, 2145.
TURCIC, M., RASIC, J. and CANIC, V. (1969) *Milchwissenschaft*, **24**, 277.
UEMURA, H., YAMADA, M., TOBA, T. and ITOH, T. (1994) *Dairy Science Abstracts*, **56**, 271.
ULBERTH, F. (1991) *Journal of AOAC International.* **74**, 630.
ULBERTH, F. and KNEIFEL, W. (1992) *Milchwissenschaft*, **47**, 432.
UMANSKII, M.S., MATVEEVA, S.K., BOROVKOVA, A. and OSTROUMOV, L.A. (1974) *XIX International Dairy Congress*, **IE**, 337.
VAITHEESWARAN, N.I. and BHAT, G.S. (1988) *Journal of Dairy Research*, **55**, 443.
VANDERPOORTFN, R. and VON RENTERGHEM, R. (1974) *XIX International Dairy Congress*, **IE**, 573.
VARMANEN, P., STEELE, J.L. and PALVA, A. (1994) *FEMS Microbiology Letters*, **124**, 315.
VARMANEN, P., RANTANEN, T., PALVA, A. and TYNKKYNEN, S. (1998) *Applied and Environmental Microbiology*, **64**, 1831.
VEDAMUTHU, E.R. (1982) United States Patent Application, US 4339464.
VESANTO, E., VARMANEN, P., STEELE, J.L. and PALVA, A. (1994) *European Journal of Biochemistry*, **224**, 991.
VESCOVO, M., TORRIANI, S., SCOLARI, G. and DICKS, L.M.T. (1995) *Annali di Microbiologia ed Enzimologia*, **45**, 51.
VIANI, R. and HORMAN, I. (1976) In *Nestlé Research News 1974/75*, Ed. by Boella, C., Nestlé Products Technical Assistance, Lausanne, pp. 53–54.
VINALS, C., DE BOLLE, X., DEPIEREUX, E. and FEYTMANS, E. (1995) *Proteins: Structure, Functions and Genetics*, **21**, 307.
WACHER-RODARTE, C., GALVAN, M.V., FARRES, A., GALLARDO, F., MARSHALL, V.M.E. and GARCIA-GARIBAY, M. (1993) *Journal of Dairy Research*, **60**, 247.
WACHOL-DREWEK, Z. and ROCZNIAK, B. (1982) *XXI International Dairy Congress*, Vol. 1, Book 1, 309.
WALSTRA, P. and JENNESS (1984) In *Dairy Chemistry and Physics*, John Wiley & Sons, New York.
WANG, D., SKAKIBARA, M., KONDOH, N. and SUZUKI, K. (1996) *Journal of Chemical Technology and Biotechnology*, **65**, 86.
WEIHRAUCH, J.L. (1988) In *Fundamentals of Dairy Chemistry*, 3rd Edition, Ed. by Wong, N.P., Van Nostrand Reinhold, New York, pp. 215–278.
WEIMER, K., OBERG, C.J., MOYES, L.V., BROWN, R.J. and RICHARDSON, G.H. (1989) *Journal of Dairy Science*, **72**, 2873.
WIGERTZ, K., SVENSSON, U.K. and JÄGERSTAD, M. (1997) *Journal of Dairy Research*, **64**, 239.
WILKINS, D.W., SCHMIDT, R.H. and KENNEDY, L.B. (1986a) *Journal of Agriculture and Food Chemistry*, **34**, 150.
WILKINS, D.W., SCHMIDT, R.H., SHIREMAN, R.B., SMITH, K.L. and JEZESKI, J.J. (1986b) *Journal of Dairy Science*, **69**, 1219.
WINTERS, D.A. and BATT, C.A. (1991) *Milchwissenschaft*, **46**, 753.
WOHLRAB, Y. and BOCKELMANN, W. (1992) *International Dairy Journal*, **2**, 345.
WOHLRAB, Y. and BOCKELMANN, W. (1993) *International Dairy Journal*, **3**, 685.
WOHLRAB, Y. and BOCKELMANN, W. (1994) *International Dairy Journal*, **4**, 409.
WOLIN, M.J. (1964) *Science*, **146**, 775.
YAMAMOT, N., AKINO, A. and TAKANO, T. (1993) *Journal of Biochemistry*, **114**, 740.
YAMAMOT, N., AKINO, A. and TAKANO, T. (1994) *Journal of Dairy Science*, **77**, 917.
YAYGIN, H. (1982a) *XXI International Dairy Congress*, Vol. 1, Book 1, 294.
YAYGIN, H. (1982b) *XXI International Dairy Congress*, Vol. 1, Book 1, 302.
YAYGIN, H. and MEHANNA, N.M. (1988) *Indian Journal of Dairy Science*, **41**, 432.
YOAST, S., ADAMS, R.M., MAINZER, S.E., MOON, K., PALOMBELLA, A.L. and SCHMIDT, B.F. (1994) *Applied and Environmental Microbiology*, **60**, 1221.
YU, J.H. and CHUNG, E.Y. (1986) *Dairy Science Abstracts*, **48**, 340.
YU, J.H. and NAKANISHI, T. (1975a) *Japanese Journal of Dairy Science*, **24**, A27.
YU, J.H. and NAKANISHI, T. (1975b) *Japanese Journal of Dairy Science*, **24**, A79.
YU, J.H. and NAKANISHI, T. (1975c) *Japanese Journal of Dairy Science*, **24**, A117.
YU, J.H., NAKANISHI, T. and SUYAMA, K. (1974) *Japanese Journal of Dairy Science*, **23**, A195.

YU, J.H., SAITO, M. and LEE, K.H. (1985) *Dairy Science Abstracts*, **47**, 642.
ZANATTA, P. and BASSO, A. (1992) *Lait*, **72**, 285.
ZEVACO, C. and GRIPON, J.-C. (1988) *Lait*, **68**, 393.
ZOURARI, A. and DESMAZEAUD, M.J. (1990) In *Processing and Quality of Foods*, Vol. 2, Ed. by Zeuthen, P., Cheftel, J.C., Eriksson, C., Gormley, T.R., Linko, P. and Paulus, K., Elsevier Applied Science, London, pp. 2.46–2.51.
ZOURARI, A. and DESMAZEAUD, M.J. (1991) *Lait*, **71**, 463.
ZOURARI, A., ROGERS, S., CHABANET, C. and DESMAZEAUD, M.J. (1991) *Lait*, **71**, 445.
ZOURARI, A., ACCOLAS, J.-P. and DESMAZEAUD, M.J. (1992a) *Lait*, **72**, 1.
ZOURARI, A., COMMISSAIRE, J. and DESMAZEAUD, M.J. (1992b) *Journal of Dairy Research*, **59**, 105.